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TESIS DE DOCTORADO

DETECTION AND GENOTYPING OF HUMAN SAPOVIRUS IN CLINICAL AND SHELLFISH SAMPLES FROM GALICIA

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Detection and genotyping of human sapovirus in clinical and shellfish samples from Galicia

D. Miguel Ángel Fernández Varela,

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Detection and genotyping of human sapovirus in clinical and shellfish samples from Galicia

D. Jesús López Romalde

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"Hay una fuerza motriz más poderosa que el vapor,
la electricidad y la energía atómica:
la voluntad"
(Albert Einstein, 1879 -1955)

"En el campo de la investigación, el azar no favorece
más que a los espíritus preparados"
(Louis Pasteur, 1822-1895)

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A mi padre, a mi madre y a mi hermana.

A Alba.

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PUBLICATIONS

Publications derived from the present doctoral thesis:

Varela MF, Polo D, Romalde JL. (2016) Prevalence and genetic diversity of human sapoviruses in shellfish from commercial production areas in Galicia, Spain. *Applied and Environmental Microbiology*, 82:1167-1172. Journal Impact Factor 2016: 3.807. Q1, 29/124 in Microbiology.

Varela MF, Hooper AS, Rivadulla E, Romalde JL. (2016) Human sapovirus in mussels from ría do Burgo, A Coruña (Spain). *Food and Environmental Virology*, 8:187-193. Journal Impact Factor 2016: 1.847. Q2, 113/229 in Environmental Science.

Romalde JL, Manso CF, Varela MF, Rivadulla E, Polo D. (2017) Enfermedades de transmisión alimentaria: infecciones por Norovirus y virus de la hepatitis A. In: *El cultivo de la ostra rizada en Galicia. Pasado, Presente y Futuro.* Xunta de Galicia / CETMAR, Vigo, Spain. ISBN: 978-84-697-3753-8.

Romalde JL, Rivadulla E, Varela MF, Barja. (2017) An overview of 20 years of studies on the prevalence of human enteric viruses in shellfish from Galicia, Spain. *Journal of Applied Microbiology*, 124:943-957. Journal Impact Factor 2016: 2.099. Q3, 78/124 in Microbiology.

Varela MF, Monteiro S, Rivadulla E, Santos R, Romalde JL. (2018) Development of a novel digital RT-PCR method for detection of human sapovirus in different matrices. *Journal of Virological Methods*, 254:21-24. Journal Impact Factor 2016: 1.693. Q3, 101/160 in Biotechnology & Applied Microbiology.

Varela MF, Rivadulla E, Lema A, Romalde JL. (2018) Human sapovirus among outpatients with acute gastroenteritis in Spain: a one year study. *Clinical Microbiology and Infection*, (Submitted). Journal Impact Factor 2016: 5.292. Q1, 8/84 in Infectious Diseases (top decile).

Other publications related with the present doctoral thesis:

Polo D, Feal X, Varela MF, Monteagudo A, Romalde JL. (2014) Depuration kinetics of murine norovirus in shellfish. *Food Research International*, 64:182-187. Journal Impact Factor 2014: 2.818. Q1, 21/129 in Food Science & Technology.

Polo D, Varela MF, Romalde JL. (2015) Detection and quantification of hepatitis A virus and norovirus in Spanish authorized shellfish harvesting areas. *International Journal of Food Microbiology*, 193:43-50. Journal Impact Factor 2015: 3.445. Q1, 13/129 in Food Science & Technology.

Mesquita JR, Oliveira D, Rivadulla E, Abreu-Silva J, Varela MF, Romalde JL, Nascimento MSJ. (2016). Hepatitis E virus genotype 3 in mussels (*Mytilus galloprovinciallis*), Spain. *Food Microbiology*, 58:13-15. Journal Impact Factor 2016: 3.759. Q1, 9/129 in Food Science & Technology (top decile).

Rivadulla E, Varela MF, Romalde JL. (2017) Low prevalence of Aichi virus in molluscan shellfish samples from Galicia (NW Spain). *Journal of Applied Microbiology*, 122:516-521. Journal Impact Factor 2016: 2.099. Q3, 78/124 in Microbiology.

Varela MF, Ouardani I, Kato T, Kadoya S, Aouni M, Sano D, Romalde JL. (2018) Sapovirus in wastewater treatment plants in Tunisia: prevalence, removal, and genetic characterization. *Applied and Environmental Microbiology*, 84:e02093-17. Journal Impact Factor 2016: 3.807. Q1, 29/124 in Microbiology.

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THESIS INDEX

ABBREVIATIONS & ACRONYMS

°C	Celsius degree
AdV	adenovirus
AiV	aichivirus
aa	amino acid
AsV	astrovirus
cm^3	cubic centimetre
CoV	coronavirus
CoxV	coxsackievirus
DNA	deoxyribonucleic acid
ds	double stranded
EchV	echovirus
EM	electron microscopy
ELISA	enzyme-linked immunosorbent assay
EU	European Union
EWS	early warning system
FAO	Food and Agriculture Organization of the United Nations
FBD	foodborne diseases
GC	genome copies
HAV	hepatitis A virus
HBGA	histo-blood group antigen
HEK	human embryonic kidney
HEV	hepatitis E virus
HPAI	highly pathogenic avian influenza
ICTV	International Committee on the Taxonomy of Viruses
IEM	immune electron microscopy
kb	kilobase
kDa	kiloDalton
MPa	megaPascal
MPN	most probable number
MV	Manchester virus
NGS	next generation sequencing
nm	nanometre

NoV	norovirus
NS	non-structural protein
NTPase	nucleoside-triphosphatase
ORF	open reading frame
PCR	polymerase chain reaction
pfu	plaque forming unit
PoSaV	porcine sapovirus
PV	poliovirus
qPCR	real-time/quantitative PCR
RdRp	RNA-dependent RNA polymerase
RIA	radioimmunoassay
RNA	ribonucleic acid
RT	reverse transcription
RV	rotavirus
SARS	severe acute respiratory syndrome
SaV	sapovirus
SRSV	small round structured virus
ss	single stranded
$TCID_{50}$	50% tissue culture infective dose
UK	United Kingdom
USA	United States of America
VLP	virus-like particle
vol/vol	volume-volume
VP1	major capsid protein
VP2	minor capsid protein
VPg	virus protein genome linked
WHO	World Health Organization
WWTP	wastewater treatment plant

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SUMMARY

The present doctoral thesis brings novel information on the study of human sapovirus, an emerging enteric virus causing acute gastroenteritis worldwide. This investigation was focused on the prevalence, quantification and genetic diversity observed in Galicia, Spain. With this purpose, collected shellfish samples from harvesting areas in the rías were employed, as well as clinical stool samples of people suffering acute gastroenteritis in the metropolitan area of A Coruña. Results obtained shown a significant presence of this human pathogen in shellfish and among the population. Besides, a novel digital PCR protocol to detect and quantify human sapovirus was developed, in order to improve the actual molecular techniques.

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RESUMEN

La presente tesis doctoral aporta nueva información en el estudio de sapovirus humanos, un virus entérico emergente que causa gastroenteritis aguda en todo el mundo. Esta investigación se centró en la prevalencia, cuantificación y diversidad genética en Galicia, España. Con este propósito, se recolectaron muestras de moluscos bivalvos en zonas de cultivo de las rías gallegas, así como muestras clínicas de heces de pacientes afectados por gastroenteritis aguda en el área metropolitana de A Coruña. Los resultados obtenidos muestran una significativa presencia de este patógeno humano en moluscos y en la población. Además, se llevó a cabo la puesta a punto de un nuevo protocolo de PCR digital para detectar y cuantificar sapovirus humanos y así mejorar las actuales técnicas moleculares.

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RESUMO

A presente tese doutoral aporta nova información no estudo de sapovirus humáns, un virus entérico emerxente que causa gastroenterite aguda en todo o mundo. Esta investigación centrouse na prevalencia, cuantificación e diversidade xenética en Galicia, España. Con este propósito, mostras de moluscos bivalvos foron recollidas de áreas de cultivo nas rías galegas, así coma mostras clínicas de feces de pacientes afectados por gastroenterite aguda na área metropolitana da Coruña. Os resultados obtidos mostran unha significativa presenza deste patóxeno humán en moluscos e na poboación. Ademais, levouse a cabo a posta a punto dun novo protocolo de PCR dixital para detectar e cuantificar sapovirus humáns e así mellorar as actuais técnicas moleculares.

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RESUMEN EXTENDIDO

Las enfermedades de transmisión alimentaria están causadas por una amplia variedad de agentes, y constituyen un problema de salud a nivel mundial. Ocurren como consecuencia de la ingesta de alimentos o bebidas que contienen ciertos productos químicos o microorganismos patogénicos. Los signos más comunes se presentan de forma gastrointestinal, ya sean náuseas, vómitos o diarrea. Sin embargo, también existen otras dolencias más graves, como fallos renales y hepáticos, o desórdenes neuronales, que en ocasiones pueden dar lugar a episodios de mortalidad.

Sin importar el tipo de agente causante de diarreas (mayoritariamente parásitos, bacterias o virus), muchos factores contribuyen hoy en día a la propagación de las enfermedades de transmisión alimentaria, como por ejemplo el rápido crecimiento poblacional y el comercio de alimentos entre países (algunos de ellos sin los apropiados controles microbiológicos). Además, los datos epidemiológicos siguen siendo escasos en muchos países en desarrollo, lo que sumado al carácter autolimitante de muchas de estas infecciones hace que la mayoría de los brotes pasen totalmente desapercibidos por los servicios sanitarios y las autoridades competentes. Las estimaciones apuntan a que alrededor de 23 millones de personas contraen anualmente enfermedades de transmisión alimentaria en la Unión Europea y los países

circundantes, siendo *Campylobacter spp.* y norovirus los agentes etiológicos más comúnmente identificados.

El acceso a fuentes de alimento y agua lo suficientemente saludables es una necesidad básica humana. A pesar de que todo el mundo está expuesto a los riesgos asociados a estas enfermedades, son las personas en países en desarrollo los más vulnerables. Es especialmente en esas zonas donde estas enfermedades causan una importante carga en los sistemas de salud, en el comercio y en el turismo, reduciendo la productividad económica de las familias y los negocios, e incluso amenazando en algunos casos su supervivencia.

A lo largo de la historia, muchas son las especies de virus que se han beneficiado de los alimentos y las bebidas como vehículos para infectar a humanos mediante la ruta fecal-oral. Desde principios del siglo XX han sido descritos brotes de poliomielitis causados por poliovirus, hasta que el desarrollo de su cultivo *in vitro* a mediados del pasado siglo trajo consigo la obtención de vacunas y la práctica erradicación del patógeno. En los años 40 se descubrieron los primeros casos de hepatitis víricas (virus de la hepatitis A y virus de la hepatitis E) y durante las siguientes décadas se puso de manifiesto la importancia de los moluscos bivalvos como vectores en la transmisión de virus entéricos, con la aparición de los primeros brotes asociados al consumo (crudo o poco cocinado) de estos animales.

Revisiones académicas acerca de las enfermedades de transmisión alimentaria publicadas en la segunda mitad del siglo XX llegaron a la conclusión de que las infecciones bacterianas no podían explicar la mayoría de los episodios clínicos de vómitos y gastroenteritis agudas relacionados, en muchos casos, con la ingesta de alimentos y bebidas. Los avances a finales del siglo XX en microscopía electrónica, técnicas de inmunoensayo y moleculares permitieron el descubrimiento de nuevos virus (p.ej., adenovirus, rotavirus, astrovirus, enterovirus, kobuvirus, etc.), algunos de los cuales presentan transmisión zoonótica (p.ej., coronavirus causante de síndrome respiratorio agudo grave, y la gripe aviar).

Sorprendentemente, los criterios para el control de la calidad microbiológica de los alimentos siguen dependiendo en la mayoría de los casos de estándares basados en bacterias coliformes como indicadores de la contaminación fecal. En consecuencia, el verdadero impacto de los virus entéricos en la población permanece infravalorado.

En general, los virus entéricos presentan unas características comunes que les permiten permanecer infectivos por largos periodos en diferentes situaciones y ambientes, lo cual favorece su eficiente transmisión mediante la ruta fecal-oral. Salvando ciertas excepciones, la mayor parte de las especies presentan un pequeño tamaño (de entre 27 y 35 nm de diámetro), con una cadena sencilla de RNA de sentido positivo, y sin envoltura lipídica. Así mismo, la ingesta de

unas pocas partículas virales infectivas implica una elevada probabilidad de cursar la enfermedad. El daño causado en los enterocitos del intestino delgado reduce la capacidad absortiva y digestiva, provocando la aparición de episodios de diarrea. La excreción del patógeno en las heces puede alcanzar valores de más de 10^{11} partículas virales por gramo de deposición.

Uno de los problemas fundamentales a la hora de intentar controlar la incidencia de los virus entéricos en la población, se debe a que los tratamientos de depuración de aguas residuales son en muchos casos inexistentes o ineficaces frente a estos agentes. Debido a ello, la contaminación fecal de las zonas costeras puede llevar implícita la presencia de especies virales patógenas para la población humana. Las diferentes características del ambiente acuático en el que se encuentren (p.ej., hidrografía, parámetros fisicoquímicos y biológicos, etc.) determinarán en gran medida la abundancia y diversidad de los virus presentes. En muchas ocasiones, las descargas fecales ocurren en zonas de crecimiento y cultivo de moluscos bivalvos, las cuales filtran del agua las sustancias necesarias para su nutrición. Durante este proceso de filtrado, los virus entéricos pueden ser entonces acumulados y retenidos en diferentes tejidos del animal. Esta bioacumulación convierte a las especies destinadas al consumo humano en alimentos de riesgo para la salud, debido a la resistencia ambiental del patógeno y la forma de consumo de estos alimentos, crudos o poco cocinados. Además, la clasificación de las

áreas de producción de estas especies por parte de la Unión Europea está basada únicamente en la presencia de bacterias fecales.

El objetivo principal de la presente tesis doctoral fue la adquisición de mayor conocimiento acerca de sapovirus humanos, un importante virus entérico emergente para el cual existe una gran ausencia de estudios.

Desde un punto de vista epidemiológico, el virus de la hepatitis A y norovirus son los virus entéricos más importantes transmitidos por la ruta fecal-oral, lo que deja a otros patógenos virales infravalorados y en un segundo plano. El género *Sapovirus* se clasifica dentro de la familia *Caliciviridae*, y presenta todas las características típicas previamente mencionadas para los virus entéricos: pequeño tamaño (30-38 nm de diámetro), cadena sencilla de RNA de sentido positivo (aproximadamente de 7,5 kb), y ausencia de envoltura lipídica. Además, es un virus no cultivable, y su estructura icosaédrica se observa al microscopio electrónico con una morfología en forma de estrella de David, lo que permitió su diferenciación de otros virus en los primeros estudios de identificación. El nombre (especie tipo: *Sapporo virus*) le fue acuñado debido al elevado número de análisis (y al posible descubrimiento) llevados a cabo en muestras clínicas a finales de la década de los 70 en la ciudad japonesa de Sapporo, en la prefectura de Hokkaidō.

El último informe del Comité Internacional en Taxonomía de Virus (*International Committee on Taxonomy of Viruses*) en el que

aparece sapovirus data del 2011 y clasifica al género en cinco genogrupos (del GI al GV) en base a la secuencia de la región de la proteína principal de la cápside (VP1). En base a esta clasificación, los genogrupos humanos comprenden GI, GII, GIV y GV, con 16 genotipos; e infectando el genogrupo GIII a especies porcinas. Los análisis filogenéticos llevados a cabo durante los últimos años han revelado un total de catorce nuevos genogrupos que añadir al género (del GVI al GXIX), de los cuales se incluirían cepas que infectan también a visón, perro, murciélago y rata. Desafortunadamente, el último informe oficial aún no ha publicado el capítulo correspondiente a la familia *Caliciviridae* para confirmar estas propuestas.

Hasta la realización de los estudios llevados a cabo en esta tesis, la detección de sapovirus humanos en muestras clínicas en España había sido meramente anecdótica. Por otra parte, este virus no había sido previamente identificado fuera de Japón en moluscos cultivados. Todo ello aporta novedad e innovación a los análisis desarrollados en este trabajo. Además, los avances en metodologías moleculares han hecho posible el diseño de nuevas herramientas para su estudio, como por ejemplo la aparición de la PCR digital. Las limitaciones de algunas técnicas moleculares extensamente empleadas (como es el caso de la PCR en tiempo real) pueden ser ahora mejoradas usando los protocolos de PCR digital detallados en esta tesis para la detección y cuantificación de sapovirus en los tipos

de muestra más comúnmente utilizados en investigación de virus entéricos.

Los ensayos llevados a cabo en este trabajo han seguido las directrices especificadas por las ISO correspondientes (con ligeras modificaciones), las cuales están originalmente referidas al estudio del virus de la hepatitis A y norovirus en muestras de alimentos y aguas. Estas especificaciones incluyen controles para calcular las eficiencias de extracción, y así evaluar la posible pérdida de carga viral durante el procesado de la muestra y la extracción del material genético; y las eficiencias de amplificación, utilizadas para estimar la presencia de inhibidores de la polimerasa en la muestra y evitar falsos negativos en los resultados. Todo ello aporta consistencia en los datos obtenidos y aporta seguridad y reproducibilidad para los futuros análisis e interpretaciones.

Los primeros dos artículos aquí presentados (*Article 1. Prevalence and genetic diversity of human sapoviruses in shellfish from commercial production areas in Galicia, Spain*; y *Article 2. Human sapovirus in mussels from Ría do Burgo, A Coruña (Spain)*) responden al primero de los objetivos de la tesis. Para ello evalúan la presencia y cuantificación de sapovirus humanos en diferentes especies de moluscos bivalvos cultivados en las rías gallegas mediante la técnica de RT-PCR en tiempo real. Además, se realizaron estudios filogenéticos sobre la diversidad genética, amplificando las muestras positivas por RT-PCR anillada.

Galicia, situada en la región noroeste de la Península Ibérica, está caracterizada por su peculiar topografía costera formada por rías, que suman en total casi 1.500 Km de litoral. A pocas millas al oeste del cabo de Finisterre se encuentra un importante sistema de afloramiento marino que asegura una extraordinaria productividad primaria en las rías, constituyendo un excelente ecosistema para el crecimiento y cultivo de moluscos bivalvos. Galicia constituye el 95 % de la actividad nacional en acuicultura, lo que sitúa a España dentro de los 20 países con mayor producción mundial. Entre las especies con interés comercial se encuentran almejas, berberechos, ostras y vieiras; aunque la de mayor importancia es el mejillón (*Mytilus galloprovincialis*) con más de 225.000 toneladas al año. El cultivo de esta especie se lleva a cabo en sistemas flotantes de crecimiento vertical localizados dentro de las rías, llamados bateas, de los que cuelgan cuerdas a las que se adhieren los mejillones. Este tipo de cultivo tuvo su origen a mediados del pasado siglo, en forma de negocios familiares, y hoy en día se estima que hay más de 3.300 bateas en las costas gallegas.

Los resultados obtenidos muestran una significativa prevalencia de sapovirus humanos en moluscos de Galicia. Además, se ha observado que dicha prevalencia es continua a lo largo de los periodos de estudio. Al analizar los datos de las tres rías, la presencia del virus fue positiva en 60 de las 248 muestras de moluscos analizadas (24,2 %). Los mejillones fueron la especie más

representada, y resultaron positivas 44 de las 185 muestras (23,8 %). Tanto la ría de Vigo como la ría do Burgo se encuentran bajo una enorme influencia urbana, ya que ambas están situadas al lado de dos de las ciudades más habitadas de Galicia (Vigo y A Coruña), y en ambas se detectaron sapovirus humanos: 17/78 (21,8 %) y 30/80 (37,5 %), respectivamente. En estas dos rías, la diferencia en la prevalencia viral podría estar explicada por la diferencia en tamaño entre ambas (ya que la ría de Vigo ocupa un tamaño más de tres veces mayor al de la ría do Burgo), y por tanto por las diferentes características hidrodinámicas existentes. Por otro lado, los resultados positivos de los análisis llevados a cabo en la ría de Ares-Betanzos fueron menores, con 13 de 90 muestras contaminadas con el patógeno (14,4 %). Esta ría está rodeada por áreas rurales, sin tanta influencia poblacional o industrial. De acuerdo con estas observaciones, podría existir una correlación directa entre la cantidad de habitantes viviendo en la costa y el nivel de prevalencia de sapovirus humanos en los moluscos, aunque estudios más amplios y análisis estadísticos tendrían que ser realizados para confirmar dicha hipótesis.

A día de hoy (junio de 2018), no son muchos los estudios que han analizado la presencia de sapovirus humanos en muestras de bivalvos. La mayoría de dichos estudios se realizaron en Japón y con muestreos esporádicos (no sistemáticos), y los porcentajes de prevalencia fueron muy inferiores a los obtenidos en este trabajo.

Además, muchos de ellos emplearon protocolos de PCR convencional, por lo que los datos de cuantificación son aún más limitados, siendo superiores en nuestros análisis (entre 10^3 y 10^5 copias de genoma por gramo de tejido digestivo).

Los estudios genéticos realizados para las muestras de moluscos mostraron una elevada diversidad de genotipos. De las 60 muestras positivas, 30 fueron caracterizadas, clasificándose 25 de ellas dentro del genogrupo GI. Los genotipos más comunes fueron GI.1 (11), GI.2 (10) y GI.3 (4), aunque también fueron detectadas secuencias pertenecientes a los genogrupos GII (GII.4), GIV y GV. Estos resultados concuerdan con los obtenidos por otros grupos de investigación, aunque hasta ahora este tipo de estudios son aún muy limitados.

El tercer artículo de esta tesis (*Article 3. Human sapovirus among outpatients with acute gastroenteritis in Spain: a one year study*) tuvo por objetivo analizar la incidencia de sapovirus humanos en pacientes afectados por gastroenteritis aguda en la ciudad de A Coruña, y el rol de este patógeno como agente etiológico en el área metropolitana. La metodología en este caso fue similar a los moluscos, detectando y cuantificación el virus con la técnica de RT-PCR en tiempo real, y realizando la secuenciación para los estudios filogenéticos.

La incidencia de sapovirus humanos en los pacientes con gastroenteritis agua resultó ser más elevada que en otros estudios

(15,6 %). Analizando los resultados por grupos de edad se presentan diferencias significativas, siendo los lactantes y los niños de hasta 5 años los que muestran mayores porcentajes de positivos. Además, también fue elevada la prevalencia en personas de edad avanzada. Los valores de cuantificación llegaron a superar las 10^{11} copias de genoma por gramo de deposición. Todos estos datos, junto con la estacionalidad a lo largo del estudio, coinciden en mayor medida con estudios previos.

Otro de los objetivos del trabajo fue determinar la epidemiología molecular de sapovirus humanos y su dinámica poblacional en las muestras clínicas. Además, se pretendió establecer una posible relación epidemiológica entre los episodios de gastroenteritis aguda y el consumo de moluscos bivalvos contaminados con este agente vírico. De las 186 muestras caracterizadas en las muestras clínicas, los genotipos dominantes fueron GI.1 y GI.2 (91 muestras y 32, respectivamente), lo cual coincide con los resultados de las muestras de bivalvos, y comprenden además a los dos genotipos de sapovirus humanos mayormente detectados en todos el mundo. Por otro lado, se detectaron en ambos tipos de muestras los genotipos GI.3, GII.4 y GIV.1, aunque en menor número. Estos datos apuntan hacia una clara ruta de transmisión circular: la excreción al medio ambiente de ciertas cepas de sapovirus humanos por personas infectadas, la bioacumulación de esos virus por los moluscos bivalvos cultivados en

zonas próximas, y el consumo humano de dichos animales contaminados produciendo de nuevo la enfermedad.

Para finalizar, el último de los objetivos planteados en esta tesis fue el desarrollo de una nueva metodología de PCR digital capaz de llevar a cabo análisis precisos en las muestras con matrices más comunes, para mejorar el estudio de sapovirus humanos utilizando las técnicas moleculares más novedosas. Las limitaciones de las actuales metodologías de PCR imposibilitan el establecimiento de una clara relación entre los resultados (en copias de genoma) y los virus infectivos implicados, debido a que solo tienen en cuenta la amplificación de ácidos nucleícos y los sapovirus humanos no son cultivables para estudios de infectividad. Por tanto, el riesgo real de infección sigue siendo, en parte, desconocido.

En el último de los artículos de esta tesis (*Article 4. Development of a novel digital RT-PCR method for detection of human sapovirus in different matrices*) se desarrolló un nuevo método de RT-PCR digital utilizando material estándar obtenido acorde con las especificaciones ISO. Esta técnica obtiene las cuantificaciones sin necesidad de curvas estándar, proporcionando resultados de cuantificación absoluta, y no relativa. Además, la tolerancia a la presencia de posibles inhibidores en la muestra es mucho mayor, ya que la mezcla de la reacción de amplificación se divide en miles de nano-reacciones independientes y paralelas. Los cebadores y sonda empleados fueron los mismos que los utilizados

en la RT-PCR en tiempo real, que amplifican una región parcial de la proteína de la cápside, detectando los genogrupos humanos. Se realizó también una validación analítica empleando muestras con diferentes tipos de matrices (clínicas, moluscos bivalvos y aguas ambientales), y los resultados mostraron una gran efectividad y repetitividad, así como una mayor sensibilidad que los ensayos de RT-PCR en tiempo real realizados en paralelo.

Las pasadas décadas de investigación han contribuido enormemente a los avances en el campo de la Virología. Estos avances incluyen la mejora en las técnicas de análisis y la caracterización de virus no cultivables, así como el descubrimiento de especies zoonóticas y la identificación de los agentes más importantes para la salud humana. Ha quedado también de manifiesto la clara ineficacia de los mecanismos actuales empleados para el control microbiológico en respuesta a las vulnerabilidades que amenazan el consumo de alimentos y bebidas a lo largo del globo. Futuras investigaciones más exhaustivas serán necesarias para seguir comprendiendo la presencia y distribución de sapovirus humanos en muestras de moluscos bivalvos en Galicia y su implicación en los casos de pacientes con gastroenteritis aguda. Por lo pronto, la información recabada aquí revela, una vez más, la importancia para el consumo de alimentos de unas clasificaciones, controles y límites que atiendan a la presencia de patógenos virales, y no únicamente a indicadores bacterianos. Esperamos que los

descubrimientos alcanzados en esta tesis doctoral puedan ser de ayuda llenando un hueco más de conocimiento en este campo de estudio.

1. INTRODUCTION

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1. INTRODUCTION

1.1. Foodborne diseases

Safe food saves lives. Foodborne diseases (FBD) encompass a wide spectrum of illnesses, becoming a growing problem for public health worldwide. They occur as a result of the ingestion of foodstuff or drinking water with certain chemicals or pathogenic microorganisms. The contamination may take place at different stages in the process from production to consumption ("farm to fork") by an environmental source, including pollution of water, soil or air. The most common signs of FBD present the form of gastrointestinal symptoms: nausea, vomiting and diarrhea. However, they can range from these, normally mild self-limiting, to debilitating and life-threatening diseases (kidney and liver failure, brain and neural disorders, paralysis and potentially cancers), even leading to long periods of work absenteeism as well as mortality episodes. Diseases causing diarrhea are, most of the times, caused by parasites, bacteria and viruses, becoming during the last years the 8th major cause of human deaths, killing an estimated 2.2 million people annually, and being the cause of 9% of mortalities in children worldwide (WHO, 2017).

Regardless of the pathogen, multiple factors have contributed or are contributing nowadays to FBD. The rapid population increase

on Earth and an expanding worldwide trade of food supplies and farm animals (sometimes from countries without appropriate microbiological safety procedures) are considered among the most important factors. Transport logistics and conditions have improved with the globalization, which is very efficient for the maintenance of foodstuff quality, but also enables pathogen agents to survive longer and reach the consumer in a viable form. Apart from that, human population carries its intestinal microbiota worldwide, and changes eating habits, such as the consumption of raw or lightly cooked food. Related to this, the increasing challenges include the awareness of the health impact of antimicrobial resistance in foodborne pathogens and the transmission of those resistance genes among microorganisms. Finally, climate change can bring novel vectors into different temperate regions or temperature-associated changes in contamination levels (Newell *et al.*, 2010).

Epidemiological data and full perspective on the burden of FBD remain scarce, particularly in the developing areas, and limited to a few pathogens in just some industrialized countries, because even the most noticeable foodborne outbreaks often go unrecognized, unreported or uninvestigated, and may only be visible if connected to major public health or economic impact. Baseline surveillance data is essential to observe changing levels in FBD. Besides, precise information is needed to adequately inform policy-

makers and allocate appropriate resources for food safety control and intervention efforts.

Estimations appoint that 9.4 million FBD cases in the United States of America (USA) are caused each year by 31 pathogens, resulting in more than 50,000 hospitalizations and 1,000 deaths (Scallan *et al.*, 2011a). More than 30 million additional episodes were caused by unspecified agents without sufficient data to estimate agent-specific illness (Scallan *et al.*, 2011b). Across the European Union (EU) and surrounding countries, about 23 million people become ill every year because FBD, counting approximately 3,000 mortalities, being *Campylobacter spp.* and norovirus (NoV) the most common infective agents; even more, the occurrence of the outbreaks have not stopped increasing since the first stages of the 21st century (WHO, 2015; EFSA/ECDC, 2016).

Access to sufficient and safe food is a basic human necessity, essential for creating a world without hunger and for achieving poverty reduction for mankind. While everyone is exposed to foodborne health risks, it is the people in developing countries who are most exposed and vulnerable to these risks. Unsafe water used for the cleaning and processing of food, poor food-production processes (including inappropriate use of agricultural chemicals) and food handling, the absence of adequate food storage infrastructures, and inadequate or poorly enforced regulatory standards; all contribute to a high risk environment (WHO, 2015).

FBD not only adversely affect people's health and well-being, but also have negative economic consequences for individuals, families, communities, businesses and countries. These diseases impose a substantial burden on health-care systems, trade and tourism, they reduce economic productivity and threaten livelihood.

1.2. Foodborne enteric viruses

1.2.1. Historical perspective

The development of the microscope led to the discovery of single-cell organisms such as algae, protozoa, and bacteria. Although many of these were free-living environmental forms, it was eventually demonstrated that some of them could cause diseases (Koch, 1876 in Brock, 1961). Laboratory methods were developed for cultivation of bacteria, but these techniques were generally not applicable to infectious protozoa, and still some diseases that were clearly infectious were caused by agents smaller than bacteria. These infectious agents (*contagium vivum fluidum*) (Beijerinck, 1898 in Hahon, 1964) were shown to pass through unglazed porcelain filters that would retain bacteria. An early descriptor was "filterable virus" (Williams, 1964), and the word *virus* was originally used in Latin to denote a disease agent or a poison. Over many years it was developed the definition of viruses as having either DNA or RNA, one or more protein capsid, and, some, a lipid-containing envelope.

Methods of propagating viruses, first in whole host organisms and then in cultured cells, were essential to progress in Virology (Enders *et al*., 1949). Electron microscopy provided images of these agents, sometimes biased by technical artifacts. The development and application of molecular methods as polymerase chain reaction (PCR) assays has been a major advance in the ability to detect viruses in foods. However, removal of interfering substances, small assay volume, and determination of infectivity are areas that still require improvement. Nowadays it is used for the investigation of outbreaks, to demonstrate the virus in the implicate food, and to track sources of contamination. Meanwhile, the recognition of prions has added an even smaller infectious agent to the picture (Prusiner, 1982).

Food was first recognized as a vehicle for the transmission of viruses in 1914 when a raw milk-associated outbreak of "infantile paralysis" was reported (Jubb, 1915). Four cases happened in an English community among children who drank from a common source. The causative agent was determined to be poliovirus (PV), isolated in 1908 by Landsteiner and Popper, and therefore the disease became named poliomyelitis (*poliós*: grey; *myelós*: marrow). PV was later classified as the type species into the *Picornaviridae* (*pico*: small; *RNA*: ribonucleic acid). Additional foodborne poliomyelitis outbreaks were reported in the United Kingdom (UK) and the USA until 1949 (Enders *et al*., 1949), but with the *in vitro* PV cultivation and the development of a vaccine, no outbreaks were

reported in the industrialized world after the early 1950s (Sattar & Tetro, 2001). Raw milk was the apparent vehicle in these outbreaks and perhaps it had been contaminated after heating. Other foods suspected of having served as vehicles in these outbreaks were lemonade (Piszczek *et al*., 1941) and cream-filled pastries (Hargreaves, 1949).

The 1940s became the decade of the discovery of the first viral hepatitis outbreaks (Mosley, 1967). One in particular became very important, comprising more than 28,000 cases in New Delhi, India, in 1955 to 1956 (Dienstag, 1981). It led to the appearance of the environmental virology as the scientific discipline that is nowadays. This outbreak had not been caused by hepatitis A virus (HAV), as firstly thought, but by hepatitis E virus (HEV), according to late 20th century investigations on the episode (Marano *et al.*, 2015).

Methods for propagating, detecting, and identifying viruses were in their infancy in the early 1960s. Although there had been significant progress in clinical virology, dealing with viruses outside the host required a different mindset as specific viral agents could not be isolated *in vitro*. For the next two decades, the study of foodborne viruses focused on potential risks from the use of reclaimed wastewater for irrigation of food crops and virus transmission by shellfish consumption. Early reviews of foodborne viral illnesses, published in 1969 and 1983, concluded that bacterial infections could not explain most of the gastrointestinal diseases or

outbreaks associated with clinical episodes of vomiting and acute diarrhea, related in many cases with food intakes (Cliver, 1969; 1983). In relation to this, the first recorded shellfish-associated outbreak occurred among people eating raw oysters in Sweden in 1955 to 1956, comprising more than 600 cases of HAV (Roos, 1956), and soon afterwards it was detected in the USA too (Mason & McLean, 1962).

Although any virus capable of transmission by the fecal-oral route can be transmitted by foods, human caliciviruses, such as NoV, are believed to be the major cause of viral FBD. Caliciviruses are estimated to cause 58 % of gastroenteritis cases in USA per year (Scallan *et al.*, 2011a). Its prototype genus, NoV, received the early name of Norwalk-like virus (NLV) or small round structure virus (SRSV), as it was detected from volunteer patients infected with samples from a 1968 outbreak occurred in Norwalk, Ohio (USA) using immune electron microscopy (IEM) (Kapikian *et al*., 1972).

Advances in electron microscopy (EM) during 1970s, and further immunoassay techniques during the 80s and 90s, led to the inclusion of new members into the most important viral families related with FBD (mainly *Picorndaviridae* and *Caliciviridae*) (Madeley & Crosgrove, 1975; Kapikian *et al*., 1972; Herrmann *et al*., 1995). In the 1990s, molecular methods became available for the detection of difficult to cultivate or non-cultivable viruses, which led to the definite realization that viruses are the leading cause of FBD in the

developed world, and outbreaks caused by them are increasing worldwide (Bresee *et al*., 2002; Koopmans & Duizer, 2004).

Until not so long ago, it was thought that foodborne human enteric viruses could only be originated from humans, and hence their transmission was limited to contaminated food handlers, food or water. However, outbreaks of HEV have demonstrated that it is a zoonotic virus capable of transmission by consumption of raw or lightly cooked meat products (Mishiro, 2004; Tei *et al.*, 2004). The close relationship between human NoV and some animal caliviciviruses, such as those found in calves, suggested that interspecies transmission to humans might have been possible (Koopmans *et al*., 2002). In addition, there are several other animal viruses that have demonstrated an ability to cross species barriers and have the potential to become involved in human disease. These include severe acute respiratory syndrome (SARS) coronavirus (CoV), which was spread into the human population through the preparation and consumption of food animals that appear to have contracted the infection from another reservoir, probably bats (Lau *et al*., 2005); H5N1 highly pathogenic avian influenza viruses (HPAI), that have been transmitted from duck meat and the consumption of duck blood, resulting in the infection of humans (Tumpe *et al*., 2002); and others like picobirnaviruses, toroviruses, parvoviruses, bornaviruses, and pestiviruses.

More than a hundred years after the first studied outbreak occurred by a foodborne enteric virus, there is not any systematic surveillance on the viral etiology of the FBD. Remarkably, the microbiological quality control criteria for food still rely mostly on standard counts of coliform bacteria, developed as indicators for fecal contamination. There is now ample documentation that these criteria are insufficient to protect against viral foodborne infections (Newell *et al*., 2010). Therefore, the real impact of enteric viruses on the human population is nowadays unknown and limited to some developed countries (Morris, 2011). Epidemiological data sometimes only refer to bacterial enteric diseases and do not focus on the foodborne nature of transmission. More tools and global efforts are needed in order to establish a common frame of work in this still emerging discipline of virology.

1.2.2. General characteristics of enteric viruses

Enteric viruses do not have any metabolic activity. They just consist of either DNA, as adenovirus (AdV), or RNA, like HAV, HEV, caliciviruses, astrovirus (AsV) and rotavirus (RV), enclosed within a protective protein capsid, using the synthetic machinery of host cells to produce new viral particles.

Viruses do not replicate in food or water, they need living cells to multiply. This means that the transmission via food reflects fecal contamination, with their persistence on or in the product. Enteric

viruses persistence outside the cell host (with digestive enzymes and bile salts in the human digestive tract) is advantaged by their natural resistance to acidic conditions, which confers them longer survival periods in the environment under extreme conditions. It allows them a protection against bactericidal processes often used in food production industries and wastewater treatment plants (WWTPs). Except some species like CoV, RV and AdV, most of the enteric viruses have small size (between 27 and 35 nm of diameter), present positive sense (+) single stranded (ss) RNA and have no lipid bilayer envelope (naked viruses), (Richards, 2005).

For most of the common enteric viruses causing gastroenteritis, the ingestion of a few viable particles (10–100) has a significant probability of causing an infection. In contrast, most bacteria require of thousands of cells (Teunis *et al.*, 2008). Viral damage occurs at the enterocytes of the villi in the small intestine, reducing absorptive and digestive capacity, and causing liquid retention within the intestinal lumen. Fermentation of the undigested material increases the production of low molecular mass particles which subsequently increases the osmotic pressures within the large intestine. Once the absorptive capacity of the large intestine is exceeded, diarrhea occurs (Conner & Ramig, 1997). One might speculate that the extent of the diarrhea is proportional to the amount of enterocyte damage; however, this has not been quantitatively determined. Virus shedding in infected individuals

reach very high loads (even 10^6-10^{11} viral particles per gram of stool), spreading rapidly from one individual to the next (Farthing, 1989).

One of the fundamental problems regarding the study of viruses in food is the presence of low numbers of viruses, which requires large volumes of samples to assess the risk of infection. In addition, viruses need to be removed and extracted in order to reduce the assay volume and get rid of substances that might be toxic to cell cultures or interfere with detection by molecular methods. These limitations on viral assays make them more difficult and costly processes as compared with the detection of foodborne bacteria. Many contaminations are likely to occur on the surface by irrigation water or improper handling, with the exception shellfish, where their internal structures create additional difficulties in the recovery of the virus (Gerba, 2006).

There is a wide variety of symptoms associated to the infection by foodborne enteric viruses. In general, they can be gathered into three types of general diseases: (a) gastroenteritis, with vomits and diarrhea caused, for example, by NoV, sapovirus (SaV), RV, AsV, aichivirus (AiV) and AdV; (b) enteric hepatitis, where the principal site of virus infections is not the intestine, but rather the liver, caused by HAV and HEV migrating and manifesting abdominal pain and jaundice; and (c) a third group of viruses enclosing PV, coxsackievirus (CoxV) or echovirus (EchV), that replicate themselves in the intestine, but only cause the disease after drifting to other

organs of the body, like the eyes, the central nervous system or the respiratory system, causing illnesses such as conjunctivitis, poliomyelitis, meningitis or encephalitis.

1.2.3. Main enteric viruses

A large amount of species have been identified as etiological agents of foodborne viral diseases, belonging to 11 different families (Newell *et al.*, 2010). The two most important, under an epidemiological point of view, are *Caliciviridae* and *Picornaviridae* families, in which NoV and HAV are included, respectively; but others, like *Adenoviridae*, *Astroviridae, Hepeviridae* or *Reoviridae*, also deserve some of our attention (Table 1).

Adenoviridae

AdV was discovered in 1953 and is one of the earliest foodborne viruses studied (Rowe *et al.*, 1953). They belong in the genus *Mastadenovirus*, with 51 serotypes, of which serotypes 40 and 41 are responsible for epidemic and endemic acute gastroenteritis in children and adults. They have 34-37 kb linear double stranded (ds) DNA (Group I in Baltimore classification). AdV are non-enveloped viruses with a diameter between 70 and 110 nm with fiber-like projections on their icosahedral capsid (Lion, 2014). These viruses cause respiratory, ocular, kidney, neurological and gastroenteritis

illnesses. Enteric AdV infection is primarily caused by just a few serotypes within the species F. It may occur mostly among children in developing countries, and it is characterized by watery diarrhea, often containing mucus, fever, vomits, abdominal pain and, sometimes, respiratory illness with a mean incubation time of 10 days (Ruuskanen *et al.*, 2002).

Reoviridae

This family encompasses a large amount of genera, of which *Rotavirus*, among others, affect humans. RV genetic structure and morphology differs with other enteric viruses, with a dsRNA genome surrounded by a three-layered protein capsid and a diameter size of 78 nm, allocated into Group III according to Baltimore classification. They are divided into nine species, being the RV A one of the most commonly enteric viruses detected worldwide. RV is especially known to cause an abrupt onset of fever accompanied by vomiting and diarrhea that can elicit mortal dehydration on infants, although an effective vaccine is nowadays provided (Todd & Greig, 2015).

Astroviridae

They were firstly named after their distinctive, strellate, surface configuration (Madeley & Cosgrove, 1976) and are nowadays included into genus *Mamastrovirus*, presenting a non-enveloped morphology and polyadenylated (+)ssRNA (Group IV in Baltimore classification). General population commonly has antibodies against AsV, causing just mild episodes of gastroenteritis in humans, usually in children (LeBaron *et al.*, 1990). More serious symptoms may result in immunocompromised or severely malnourished individuals, due to dehydration.

Caliciviridae

Human caliciviruses may be divided into two genera: *Norovirus* and *Sapovirus*. Both encompass a broad range of genetically diverse genogroups and genotypes, with icosahedral non-enveloped morphology, 27 to 40 nm of diameter. They have +ssRNA, and are included, therefore, into the Group IV of the Baltimore classification. Family was named after the cup-shaped/calyx depressions of their surface observed under EM. Calicivirus illnesses on humans are characterized by acute onset of non-bloody diarrhea and/or vomiting, often accompanied by other symptoms, as nausea, abdominal cramps, chills and fever (Agus *et al.*, 1973).

Incubation periods normally range between 10 and 51 hours after virus ingestion (Green *et al.*, 2001).

NoV is composed of at least 41 genotypes within 7 genogroups (GI–GVII) of which GI, GII and GIV infect humans (Zheng *et al.*, 2006). It is considered the major non-bacterial foodborne worldwide in patients of all ages, being causative of half of all foodborne outbreaks in the USA according to the Centers of Disease Control and Prevention (Vinje, 2015). NoV associated deaths are rare, with an incidence of 0.19-0.40 fatalities per 10,000 people (CDC, 2016). It has been detected worldwide although its prevalence still remains underestimated.

SaV constitute the object of study in the present doctoral thesis and will be reviewed in the following sections.

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Table 1. General characteristics of the main enteric viruses.

Family	Genus	Type species (viruses)	Genome (Kb)	Morphology (nm)	Disease*
Adenoviridae	*Mastadenovirus*	*Human mastadenovirus C* (AdV)	dsDNA (34-37)	Icosahedral (70-110)	Respiratory, ocular, renal, neurological, gastroenteritis
Reoviridae	*Rotavirus*	*Rotavirus A* (RV)	dsRNA (16-27)	Icosahedral (78)	Gastroenteritis
Astroviridae	*Mamastrovirus*	*Mamastrovirus 1* (AsV)	(+)ssRNA (7-8)	Icosahedral (28-30)	Gastroenteritis
Caliciviridae	*Norovirus*	*Norwalk virus* (NoV)	(+)ssRNA (7.3-7.6)	Icosahedral (27-40)	Gastroenteritis
	Sapovirus	*Sapporo virus* (SaV)	(+)ssRNA (7.5)	Icosahedral (30-38)	Gastroenteritis
Hepeviridae	*Orthohepevirus*	*Orthohepevirus A* (HEV)	(+)ssRNA (7.2)	Icosahedral (32-34)	Hepatitis
Picornaviridae	*Hepatovirus*	*Hepatovirus A* (HAV)	(+)ssRNA (7.5)	Icosahedral (27-32)	Hepatitis
	Enterovirus	*Enterovirus C* (CoxV, EchV, PV)	(+)ssRNA (7.2-8.4)	Icosahedral (28-30)	Poliomyelitis, aseptic meningitis, respiratory, neurological
	Kobuvirus	*Aichivirus A* (AiV)	(+)ssRNA (8.3)	Icosahedral (30)	Gastroenteritis

*caused by species of the genus

Hepeviridae

HEV, within genus *Orthohepevirus*, is a hepatotropic non-enveloped (+)ssRNA enteric virus (Group IV in Baltimore classification). Comparative analyses of the nucleotide sequences led to the recognition of four genotypes (Purdy *et al.*, 2017). Genotypes 1 and 2 are prevalent in developing countries, whereas genotypes 3 and 4 have brought much attention due to their zoonotic transmission through the consumption of raw or undercooked pork meat (Ankcorn & Tedder, 2017). HEV is widely present in swine to such an extent that they are now considered important reservoirs for human disease (Mesquita *et al.*, 2014).

Picornaviridae

This family is included into the order *Picornavirales*, and integrates a large amount of genera, of which *Hepatovirus*, *Enterovirus* and *Kobuvirus* are worthy of highlighting. Again, all viruses belonging to this family are labeled into the Group IV of Baltimore classification for presenting (+)ssRNA as nucleic acid and an icosahedral non-enveloped morphology with an approximately diameter of 30 nm.

Belonging to genus *Hepatovirus*, HAV They have one identified serotype and six human subtypes described

nowadays, with an approximately 7.5 kb genome. They enter the vascular system through the epithelium of the oropharynx or intestine, reaching the liver and multiplying within hepatocytes and macrophages. Virions are secreted into the bile and reach back the intestine and stools. Incubation period takes 2 to 4 weeks, which makes it difficult to associate with any type of food that could cause sporadic outbreaks, leading to an underestimation of foodborne cases of HAV (Vaughan *et al.*, 2014). Typical foodborne infection is much more severe and long-lasting than gastroenteritis, with high mortality rates with fulminant hepatitis, although it can be prevented by vaccination (Todd & Greig, 2015).

Humans are the only known natural hosts of genus *Enterovirus*, which takes the main role as the foodborne viruses affecting different organs than intestine and liver. CoxV, EchV and PV (almost eradicated today) produce symptoms like paralysis, myocarditis, encephalitis, aseptic meningitis and multi-organ failure, including more than 70 serotypes and presenting an outstanding survival, with remaining infectious virus in fomites after several days when associated with bacteria and/or organic matter. At last, genus *Kobuvirus* encloses different mammal viral pathogens, being AiV the type species and another human gastroenteritis etiological agent (Todd & Greig, 2015; Zell *et al.*, 2016).

1.3. Special priority food products. Enteric viruses in bivalve molluscs

Food contamination by enteric viruses, including zoonotic infections, may occur at any phase of the production chain: culture, harvesting, processing, distribution or handling. Depending on the stage where the contamination takes place and the type of food implicated, most of the viral FBD can be related with a sector of the economy:

Primary sector: fresh food with minimum process, such as bivalve molluscs, vegetables, fruits, cereals and water, which are typically contaminated on the environment during primary production. The key factor in these cases is water quality: drinking, irrigated or waste water (as for example in bivalve molluscs grown areas).

Secondary and tertiary sectors: food products contaminated during their processing and/or handling, through infected people or fomites. Risk of contamination is closely related with hygienic conditions of workers and handlers, including in this group foods normally consumed cold, raw or lightly cooked (such as pastry, fast food, sausages, and processed salads).

Experts from the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO)

sought to identify virus commodity combinations of concern by considering both the incidence of FBD linked to a specific commodity and the evidence for those goods in causing viral FBD (WHO/FAO, 2008). The report concluded with the following main five sources of enteric viruses-commodity combinations:

A. *Caliciviruses & HAV-bivalve molluscs.* For shellfish (oysters, clams, cockles and mussels), the major well-documented route of contamination is via fecal contamination of harvesting areas, considering handling by infected individuals relatively minor relevant for this combination. Viruses have been observed to persist for several weeks in contaminated live shellfish gut tissue and their persistence after depuration can be explained, in some cases, by their specific bind to receptor sites (Le Guyader *et al.*, 2006a).

B. *Caliciviruses & HAV-fresh produce.* Contamination may occur either at the pre-harvest stage with contaminated wastewater (irrigation or agrochemical application) and/or infected pickers; or at the post-harvest phase (food handlers). Viral outbreaks associated with contaminated fresh items are well documented, as for example the one occurred in Germany, 2012, caused by NoV in imported frozen strawberries from China (Bernard *et al.*, 2014).

C. *Caliciviruses & HAV-prepared foods.* Infected handlers are the main source of contamination for this virus-

commodity combination. Even though less important, surface contamination resulting from vomiting or contaminated foods on the same surface (cross-contamination) is also possible. In all instances, where a person carrying a virus comes into contact with food, contamination might occur, and, due to the stability of these pathogens, they are likely to survive in many foods that do not receive a heating step prior to consumption (Koopmans & Duizer, 2004).

D. *RV-water for food preparation.* Infections by this virus occur globally, as the virus is the leading cause of gastroenteritis in infants and young children worldwide, mainly in developing countries. Although person-to-person transmission is most common, there is ample documentation of the potential for virus transmission via drinking water and water used for food preparation (Villena *et al.*, 2003).

E. *Emerging viruses-selected commodities.* Foodborne outbreaks associated with emerging viruses are usually scarce but they happen to create such an important impact. Examples of these could be HPAI virus in undercooked poultry or eggs, HEV in porcine organs or muscle tissue, and SARS-CoV. All these three viruses happen to be zoonotic viruses, but limited epidemiological

data exist on outbreak events and no systematic surveillance exists that could help on an early identification.

1.3.1. Aquaculture, bivalve molluscs and Galicia

FAO defines aquaculture as the aquatic equivalent of farming but in water environments, both inland (freshwater) and coastal (brackish water and seawater) areas. It includes animals (like crustaceans, finfish and molluscs) and plants (as seaweeds and freshwater macrophytes). Aquaculture importance has increased during the 20th century as a result of the stabilization of the fishing production worldwide and the rise of global demand due to the growing population on Earth.

One of the best adapted taxa to the aquaculture intense harvesting methods are bivalve molluscs. The term “bivalve” was first used by Linnaeus in 1758 in his *Systema Naturae* to refer to animals having shells composed of two valves, from the Latin *bis*, meaning "two", and *valvae*, meaning "leaves of a door". On the other side, the Latin *molluscus*, from *mollis*, “soft”, was itself an adaptation of Aristotle's *τα μαλακά* (/ta malaká/), "the soft things". They present a bilateral symmetry, with a soft body laterally compressed and enclosed by a shell consisting of two hinged pieces. Bivalves, as a group, have no head and they lack some usual molluscan organs like the radula and the odontophore. They include clams, oysters,

cockles, mussels, scallops, and numerous other families that live in saltwater, as well as a number of freshwater ones. They all have been a food resource for humans since prehistoric ages and shells from some species were also used for barter and trade centuries ago.

The Spanish region of Galicia is situated at the northwest of the Iberian Peninsula, characterized by its peculiar coastline with almost 1,500 kilometers of littoral and a special topography with fiord-like inlets, called *rías*. In front of the Galician cliffs of Finisterre Cape lies the northern end of the Canary-Iberian Peninsula upwelling system, granting an extraordinary high primary productivity in the *rías*, which therefore constitute excellent ecosystems for shellfish growing and harvesting. According to FAO, Galician *rías* are considered as one of the most important repositories of phytoplankton in the world.

Several bivalve mollusc species are nowadays harvested in Galicia, positioning Spain among the 20 countries with more aquaculture production in the world (Galicia outcomes represent around 95% of Spanish aquaculture activity). Some of the most significant ones include clams: *Venerupis corrugate* (Gmelin, 1791), *Ruditapes decussates* (Linnaeus, 1758), *R. philippinarum* (Adams & Reeve, 1850); cockles: *Cerastoderma edule* (Linnaeus, 1758); oysters: *Ostrea edulis* (Linnaeus, 1758); and scallops: *Pecten maximus* (Linnaeus, 1758) (see MolluscaBase WoRMS http://www.marinespecies.org). But mussel, *Mytilus galloprovincialis*

(Lamarck, 1819), happen to be the most important species, as Galicia produces more than 225,000 tons per year (FAO, 2016) (Figure 1). Its culture starts with the harvesting of seed from wild populations, which are later grown on vertical systems of ropes suspended in the water by floating wooden rafts (called *bateas*) inside of the *rías*. Mussel farming is a family-owned business in Galicia that started its activity in the mid years of the past 20th century, and it is estimated nowadays in more than 3,300 of these *bateas*, held by around 2,300 families (FAO, 2018).

Figure 1. Evolution of the aquaculture production of mussels (*Mytilus galloprovincialis*) in Spain between 1960 and 2015 (Modified from APROMAR, 2017).

1.3.2. Bivalve molluscs as transmission vectors for enteric viruses

Aquatic ecosystems contain variable quantities of microbial planktonic communities constituted by microalgae, bacteria and viruses. Among them, different human pathogens can be found as a consequence of a natural presence in a marine environment, like bacteria *Vibrio parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*. The total viral concentration in non contaminated waters is estimated in 10^5-10^8 particles per mL (Pina *et al.*, 1998). Nevertheless, anthropogenic contamination discharged into the sea enormous amounts of viruses to nearby aquatic growing areas through raw or insufficiently treated wastewaters, with concentrations above 10^5 plaque forming units per liter (pfu/L). Most of the times, enteric viruses are excreted by sick people, but they may be present in the intestine of healthy humans (i.e., asymptomatics) or in the animal population (Schwartzbrod, 1995; Lees, 2000). Among them, NoV and HAV are the major concerns in shellfish-borne diseases.

Due to the filtering nature process to obtain food, bivalve molluscs living in contaminated aquatic environments can retain a large variety of microorganisms present in their growing areas. They can filtrate between 0.5 and 4 liters per hour, accumulating those microorganisms up to higher concentrations than in the surrounding growing waters (Bosch, 1998). For example, oysters under one hour of bioaccumulation in seawater containing 10^3 pfu/L of virus, can

achieve 27 times more concentration in their organisms (Mitchell *et al.*, 1966). Even more, molecular detection techniques have demonstrated that the digestive tract of these animals is where the enteric viruses mainly become accumulated, and once in there, viruses can persist and remain infective during long periods of time, even from harvesting to consumption (Romalde *et al.*, 1994). That is why results obtained from the hepatopancreas are more representative of the sample contamination, and it also reduces the presence of inhibitors for the detection molecular techniques (Le Guyader *et al.*, 2000).

Microbial risks due to bioaccumulation of pathogens (enteric viruses included) in bivalve molluscs lie on the fact that many species are consumed raw or lightly cooked, and the entire animal is eaten, including viscera with their digestive tissues. These circumstances are unique of these products and current problems for human health associated with the consumption of bivalve molluscs are recognized internationally. Epidemiological evidences point out enteric viruses as the main etiological agents transmitted by bivalve molluscs (Westrell *et al.*, 2010).

Oysters represent the principal disease vector within bivalve molluscs, followed by clams, mussels and cockles, which are normally lightly cooked, but rarely eaten raw. Scallops and razor shells, for instance, do not present similar infection risks as they are generally cooked reaching high temperatures that inactivate the possible

viruses and they only represent a small and specialized amount of the market. Nevertheless, these molluscs can be associated with other health-related incidents due to the contamination with biotoxins and marine bacteria (Bellou *et al.*, 2013).

The lack of surveillance systems for outbreaks with viral etiology affects any estimation regarding the human pathogens associated with the consumption of bivalve molluscs. Therefore, the real incidence of these illnesses stays underestimated, leaving the big picture of the problem barely captured on industrialized countries with a larger academic and surveillance capacity. As mentioned before, NoV and HAV represent the most commonly detected enteric viruses in bivalve molluscs, occurring mainly in Asia and Europe; but there are also documented outbreaks caused by HEV, RV, AsV, AiV or SaV, although at minor rates (Bellou *et al.*, 2013).

1.3.3. Aquatic environment contamination and bivalve viral uptake

Fecal contamination in the aquatic environment is a mixed result of flow, accumulation and persistence in both waters and sediments. Multiple parameters such as salinity, tides, marine currents and estuary morphology, hyaline stratification and sedimentation, weather and general oceanographic conditions influence on the intensity and the type of microorganisms present into the environment. These parameters also affect deeply on their

distribution and dilution along the seawater; and in processes like viral sedimentation, enzymatic destruction and predation by other microorganisms (Pommepuy *et al.*, 2005).

Current wastewater treatments (if present in the area) do not guarantee a total removal of viral pathogens, which therefore are continuously discharged to these environments. In fact, they are frequently detected in the effluents of WWTPs, rivers and surrounding waters (da Silva *et al.*, 2007; Iwai *et al.*, 2009; Maalouf *et al.*, 2010; Varela *et al.*, 2018). Intense urbanization of coastal regions led to leaks and spills of the (many times) inefficient and insufficient WWTPs, both due to the excess of their capacity or to overflows during adverse weather conditions (Murphy *et al.*, 1979; Morse *et al.*, 1986; Le Guyader *et al.*, 2006b; Le Guyader & Atmar, 2008). Also, the presence of farms near the rivers and coasts are normally associated with the use of slurry, manure and WWTPs´ sludge as fertilizers (Crowther *et al.*, 2002; Maalouf *et al.*, 2010; Zakhour *et al.*, 2010). All these facts together represent the main viral pollution entry pathways into seawaters. Other minor sources of enteric contamination in coastal zones are represented by the feces elimination from ships and boats, directly near bivalve mollusc production areas, that can include some of infected people (Dowell *et al.*, 1995; Gerba, 2000; Kohn *et al.*, 1995).

Once in the aquatic environment, enteric viruses´ appearance and persistence will depend on the balance between their resistance

to inactivation and different - difficult to elucidate - factors, including their degradation and distribution. Different from other microorganisms, viruses can remain stable in the environment during long periods of time, especially if linked to organic matter and/or different colloid particles. Such association mainly depends on the intrinsic properties of the virus (size, electric charge…). Viruses, therefore, can get sedimented in the shallow seabed of an estuary and persist infectious during several months, protected by the marine sediments that can be resuspended, for example during heavy storms or anthropogenic activities (Bosch, 1998; Chung & Sobsey, 1993; da Silva *et al.*, 2008) (Figure 2).

Bivalve molluscs filtrate great amounts of seawater, obtaining their food in a selective way, from different organic matter particles. First step occurs as an adsorption of potentially food particles by the mucus (entrapping also viral particles), which is continuously secreted in the gills during the seawater pumping. Chemical composition of this mucus is essentially similar in all bivalve species, like clams and oysters; and the ionic bonds in between viral particles and mucus mucopolysaccharides compounds (sulfate radicals) are suggested as the adsorption causes (di Girolamo *et al.*, 1977; Bedford *et al.*, 1978). It has been observed that mucus production depends on the amount of glycogen in the connective tissue and the gonadal development of the animal (Galtsoff, 1964).

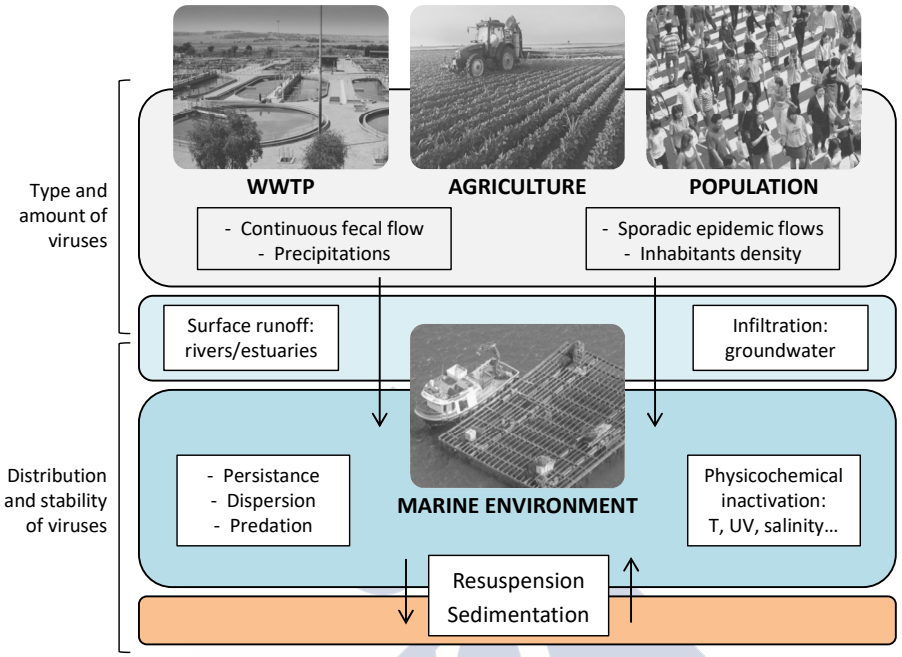

Figure 2. Origin and transmission of enteric viruses into the marine environment

Once trapped, particles (including viruses) enter the mouth to the esophagus and the stomach. From there, two different phases take place: extracellular and intracellular digestion. Extracellular digestion starts as the crystalline style rotates liberating digestive enzymes and food particles are again selected by size, density and digestibility, being transported to the intestine and eliminated through the anus in the exhaling current, or redirected to the digestive diverticula. It is there where intracellular digestion occurs and some of the viral load is driven out the animal through the feces when the digestive process ends (Figure 3).

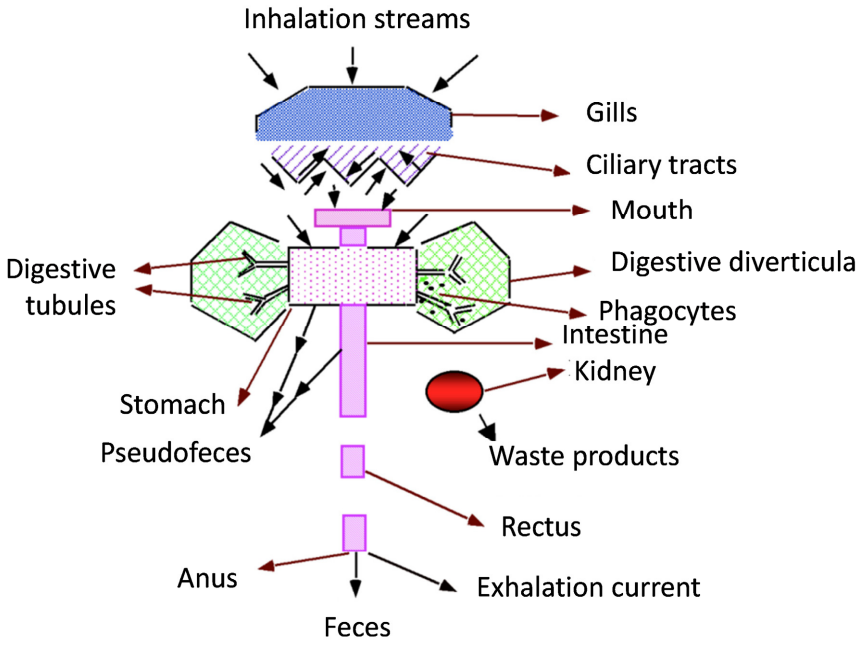

Figure 3. Scheme of filtration by bivalve molluscs.

Enteric viruses have been detected in different parts of the inner digestive system of bivalves (including connective tissues), like basal cells of the epithelium, in the lumen of the tract, in the interior of gastrointestinal cells and in hemocytes, resisting the lysosomal acid into the digestive vesicles (Romalde *et al.*, 1994; Le Guyader *et al.*, 2006a; McLeod *et al.*, 2009; Provost *et al.*, 2011). It has been discovered the existence of some antigens in the gastrointestinal cells of shellfish, similar to the human histo-blood group antigens (HBGAs), with a specific union capacity with some NoV strains. This link would occur through an alpha-type bond with a carbohydrate kind-of-structure, which has a terminal N-acetylgalactosamine

residue, equivalent to the type A human HBGA, and is considered as a potential mechanisms for viral accumulation. (Tian *et al.*, 2006; 2007). Different NoV genogroups present different union characteristics with the different structures of the human HGBA family, suggesting a possible co-evolution between NoV (mainly strains belonging to GI genogroup), its host and transmission vectors as bivalve molluscs (Le Guyader *et al.*, 2006a). Even more, epidemiological data show a predominance of NoV genogroup GI in outbreaks associated to the consumption of bivalves, while genogroup GII relates more with the contamination during handling or person-to-person (Le Guyader *et al.*, 2012). Besides, internalization of complete infectious viral particles into cells or the connective tissue could be one of the reasons of the low efficiency of the depuration processes to eliminate certain viruses, although these processes differ among virus species (Nappier et al., 2008; Polo et al., 2015).

1.3.4. Strategies for microbial control of bivalve molluscs

During the past century, various strategies have been established in shellfish growing areas throughout the world to assure the sanitary quality of shellfish. The most efficient action to avoid the contamination of bivalve molluscs is their culture in good microbiological quality seawaters. However, after harvesting treatment gives a practical option for those cases in which risks of

contamination exist. Therefore, strategies can be generally grouped into pre-harvesting: focused to improve in the water quality and to prevent on viral contamination; and post-harvesting: traditionally depuration, relocation and heat treatments, which are less efficient but practical alternatives.

Pre-harvesting

Systems for modeling the coastal environments and the early warning systems (EWSs) become helpful tools to limit or even prevent contamination (Pommepuy *et al.*, 2005). The first ones are divided into two main categories: statistical models and dynamic models. Statistical models use regression analysis of the environmental parameters and fecal contamination, predicting the microbial contamination resulting from the wastewater inputs in a specific area according to the environmental conditions. On the other hand, dynamic models were developed to overcome the limitations presented by statistical models (for instance, analyzing the type of contamination and temporal space distribution) and consist, at the same time, of different submodels, such as hydrodynamic (contamination mixture and distribution coefficients), dispersion (integrating bacteria/virus transport and diffusion) and biological processes (analyzing microbial decomposition based on environmental conditions) (Gourmelon *et al.*, 2010).

The main objective of EWSs is to obtain data in real time on production areas, such as precipitations, salinity variations, farm activities, climatic conditions, viral outbreaks upon local population, dominant mixture and transport processes… Information is immediately notified if any parameter exceeds a default value. Their function would be related with risk management, event notification and opening/closure of production areas, among others (Le Saux *et al.*, 2006; Gourmelon *et al.*, 2010).

The most efficient strategy against the presence of enteric viruses in shellfish would be to control the entrance of fecal contamination in growing areas. However, most of the wastewater treatments do not guarantee a complete removal efficiency of enteric viruses, so countries have adopted a series of sanitary controls on the production of bivalve molluscs, usually employing bacteria as indicator of fecal contamination indicators (Lees *et al.*, 2010). But, even though current policies are quite effective against the transmission of bacterial diseases, the accomplishment of those policies does not guarantee the absence of enteric viruses in the final product (Romalde *et al.*, 2002). Classification of the production zones determine the treatment that must be applied to bivalves harvested before their commercialization (depuration, relocation…). The EU and the USA (National

Shellfish Sanitation Program) base their legislations on the amount of bacterial indicators to classify each category or level. However, EU regulations (Table 2) establish the measurement of bacterial fecal indicators present in the bivalves´ flesh, while USA settles the analysis of those bacteria in seawater of each area of production (Lees *et al.*, 2010). Other developed countries have adopted mixed systems as a practical focus for both European and American market, tracing the contamination both in seawater and in bivalve tissues. The Scientific Committee on Veterinary Measures relating to Public Health issued an opinion on NoV in 2002 concluding that the conventional fecal indicators are unreliable for demonstrating the absence of NoV and that the reliance on fecal bacterial indicators removal was an unsafe practice (Anonymous, 2005).

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Table 2. EU classification criteria for categories in shellfish harvesting waters.

Production and relaying areas[1]	Units comprising the sample	Microbiological criteria limits	Analytical reference method	Post-harvesting treatment required
Class A	A pooled sample comprising a minimum of 10 individual animals	Live bivalve molluscs must not exceed, in 80 % of samples collected during the review period, 230 MPN of *E. coli* per 100 g of flesh and intravalvular liquid. The remaining 20 % of samples must not exceed 700 E. coli per 100 g of flesh and intravalvular liquid. *Salmonella* absence in 25 g	ISO TS 16649-3[2]	Live bivalve molluscs may be collected for direct human consumption
Class B	A pooled sample comprising a minimum of 10 individual animals	Live bivalve molluscs from these areas must not exceed 4,600 MPN of *E. coli* per 100 g of flesh and intravalvular liquid	ISO TS 16649-3[2]	Live bivalve molluscs may be collected, but placed on the market for human consumption only after treatment in a purification centre or after relaying so as to meet the health standards referred to in Class A
Class C	A pooled sample comprising a minimum of 10 individual animals	Live bivalve molluscs from these areas must not exceed 46,000 MPN of *E. coli* per 100 g of flesh and intravalvular liquid	ISO TS 16649-3[2]	Live bivalve molluscs may be collected but placed on the market only after relaying over a long period so as to meet the health standards referred to in Class A

Information obtained from cross references from Regulation (EC) Nº 854/2004, by Regulation (EC) Nº 853/2004, and Regulation (EC) Nº 2073/2005; all amended in Regulation (EU) 2015/2285 (Anonymous 2004a; 2004b, 2005, 2015). [1]The competent authority must classify production areas from which it authorises the harvesting of live bivalve molluscs as being of one of three categories according to the level of fecal contamination. It may, where appropriate, do so in cooperation with the food business operator. In order to classify production areas, the competent authority must define a review period for sampling data from each production. [2]Alternative methods may be used if they are validated against this reference method in accordance with the criteria in EN/ISO 16140.

Post-harvesting

Even though viral contamination might occur due to a lack of a good hygiene and handling praxis, it is demonstrated that most of viral outbreaks are related to contamination at their origin (pre-harvesting). As a consequence, viruses have been bioaccumulated and internalized inside the tissues. In these cases, product cannot be disinfected using superficial treatments, so traditionally three treatments can be applied for commercialization: depuration, relocation and heat treatment.

Depuration and relocation are two methods that take advantage of the natural pumping activity of bivalves to purge pathogenic microorganisms presented in their digestive system, reducing, in that way, the infection risk (Richards, 2001). These processes, contrary to heat treatments, allow the commercialization of alive and fresh bivalves and are worldwide used, although depuration is usually the most preferred option (Lees *et al.*, 2010).

Depuration takes place in tanks with supply of clean and disinfected seawater, under specific controlled conditions. Facilities also vary and can include open systems (continuous inputs and outputs of seawater in the installation) or closed systems (seawater is recycled through chlorination, UV radiation, ozonation and/or iodophors) (Lees, 2000; Richards, 2001). Time for commercial purpose may vary from

1 to 7 days, although generally takes around 2-3 days, and the correct handling of the animals as well as seawater parameters are crucial aspects to ensure a correct filtration and, therefore, a proper depuration (Lees *et al.*, 2010). But, even though it has been proven that enteric bacteria rapidly reduce their presence in efficient ways; enteric viruses do persist in the digestive tissues of bivalve molluscs due to the early explained process of viral accumulation in bivalves. Therefore, outbreaks associated to the consumption of depurated bivalves (fulfilling the bacterial end-product standards) have still been reported (Grohmann *et al.*, 1981; Heller *et al.*, 1986; Le Guyader *et al.*, 2006b; 2008).

On the other hand, relocation consists in the transfer of bivalve molluscs from contaminated growing areas to other cleaner marine areas. It is a long term process used as an alternative to depuration, which keeps the animals into their natural environment, making relocation a more adequate methodology when contamination levels are higher (Richards, 1988). Upon the main inconveniences are the lack of clean coastal areas with exclusive use, and the difficulties in obtaining permissions, depending on the country; plus the extra commercial cost due to additional handlings (Lees *et al.*, 2010; Richards *et al.*, 2010).

Heat treatment appears to be the most effective method to reduce the infectivity of enteric viruses in any kind of food; however it is not applicable for the commercialization of fresh products, as organoleptic changes due to the cooking process result in a lower acceptance from the consumer. Light cooking, such as steam, has a certain inactivation effect (especially when compared to raw consumption), but it is generally not enough to a complete elimination of the infectivity of the viral internalized particles present in the shellfish (Lees, 2000; Richards *et al.*, 2010).

Other methods have been proposed as alternative processes for inactivation of enteric viruses and resistant pathogens. High hydrostatic pressure studies demonstrated that a 600 MPa at 6 °C for 5 min was efficient to prevent NoV infection due to consumption of *in vitro* contaminated oysters, and the final product maintains most of its appearance, nutritional quality and organoleptic properties (Kingsley *et al.*, 2002; Leon *et al.*, 2011). Apart from that, irradiations could represent a valid option, but they have not been proved so far as useful for the viral inactivation on bivalves. UV radiation only becomes valuable when contamination occurs on the surface (de Roda Husman *et al.*, 2004), while ionizing radiation levels required would

deteriorate the organoleptic properties and increase the commercial costs (Jung *et al.*, 2009).

According to the EU Regulations on microbiological criteria for foodstuffs, fresh bivalve molluscs for direct human consumption must not exceed 230 MPN of *E. coli* per 100 g of flesh and intravalvular liquid, and, at the same time, absence of *Salmonella* in 25 g of product (Table 2). Some other countries require additional parameters to processed molluscs, for example Japan, which apart from the mentioned European standard limit, sets that total bacteria counts must not overcome 50,000 per gram and *V. parahaemolyticus* must not exceed 100 MPN/g.

1.4. Enteric viruses in consumption water

The presence of enteric viruses in water for human consumption constitutes a very special and complex issue, as it is an important transmission route for these and other pathogenic microorganisms. Therefore, low water quality still represents one of the biggest health problems. According to the WHO, the lack of clean water supplies affects mostly to children in developing countries; although it represents a water contamination problem in developed countries as well, due to the expelling by affected people via feces and the inefficiency of WWTPs in their complete elimination (da Silva *et al.*, 2007; Schuster *et al.*, 2010). The infection of people by contaminated water may also occur as a consequence of its use for

agriculture irrigation, food wash and process, the production of ice, and/or environmental exposure in recreational waters and aerosols (Koopmans *et al.*, 2002; Wyn-Jones *et al.*, 2011).

Treated drinking water may be contaminated with viruses through sewage from WWTPs, runoffs from farmlands, homes and yards, leakages of pipes, floods and discharges from industrial activities, as well as defective well casings (Chapron *et al.*, 2000). In most developing countries, most potable water pipes are made of plastic and are often subjected to physical damage, aggravated by poor urban planning and drainage systems, which predisposes treated water to eventually become contaminated with wastewater (Dongdem *et al.*, 2009).

Outbreaks can even be caused by tap and bottled water contaminated with viruses in spite of compliance with water treatment procedures, such as the recent occurred in the Barcelona metropolitan area, in Spain. In this case, more than 4,000 people were reported to suffer from gastroenteritis due to bottled water that complied with all requirements of the European Commission directive on the exploitation and marketing of natural mineral waters, but these requirements do not include any viral determination. This study concluded with the suggestion that the management of microbial risks of commercially produced mineral waters (universally based solely on bacterial parameters) could benefit from additional analysis for relevant viral pathogens, such as

NoV. However, the substantial costs incurred in developing, enhancing, and managing virus surveillance systems call for a balanced approach to keep both the cost and the time required for the analyses within feasibility limits (Blanco *et al.*, 2017).

1.5. Sapovirus

1.5.1. History

In 1976, Madeley & Cosgrove (1976) and Flewett & Davies (1976) were the first ones detecting caliciviruses viral particles in stools of children suffering gastroenteritis, using the recently introduced techniques of direct EM. Based on these studies, they were able to identify the six calyx form depressions surrounding a seventh central hollow, with an approximately 30 nm diameter, which distinguished them from AsV. Some of those findings were probably related with what we nowadays know as SaV. During the next years it was verified the relation between these enteric viruses and outbreaks and sporadic cases occurred in different parts across the globe, like Norway (Kjeldsberg, 1977), Canada (Spratt *et al.*, 1978), Japan (Suzuki *et al.*, 1979), and even associating them, in the United Kingdom, with the well known winter vomiting disease (McSwiggan *et al.*, 1978).

By then, Chiba and collaborators published in 1979 a study about an outbreak occurred two years before in an infant home in

the city of Sapporo, Japan, characterized by acute gastroenteritis and, to a lesser extent, vomiting and fever. Viral particles presented the typical “Star of David” morphology under EM and were found in fecal samples of both symptomatic and asymptomatic individuals (Chiba *et al.*, 1979) (Figure 4). The infections at the infant home moved in time from one room to another, suggesting a clear person-to-person spread, and a relationship between viral fecal shedding and the day of illness was demonstrated. Also, subclinical infections were detected using IEM techniques. This mentioned study, and further works related with the same Japanese infant home (Chiba *et al.*, 1980; Sakuma, 1981; Sakuma *et al.*, 1981), concluded, again by IEM, that the virus appeared to have no antigenic relationship to any other gastroenteritis candidate virus at the time, leading to the appointment of the virus, in a first moment, as “Sapporo agent” (Kogasaka *et al.*, 1981).

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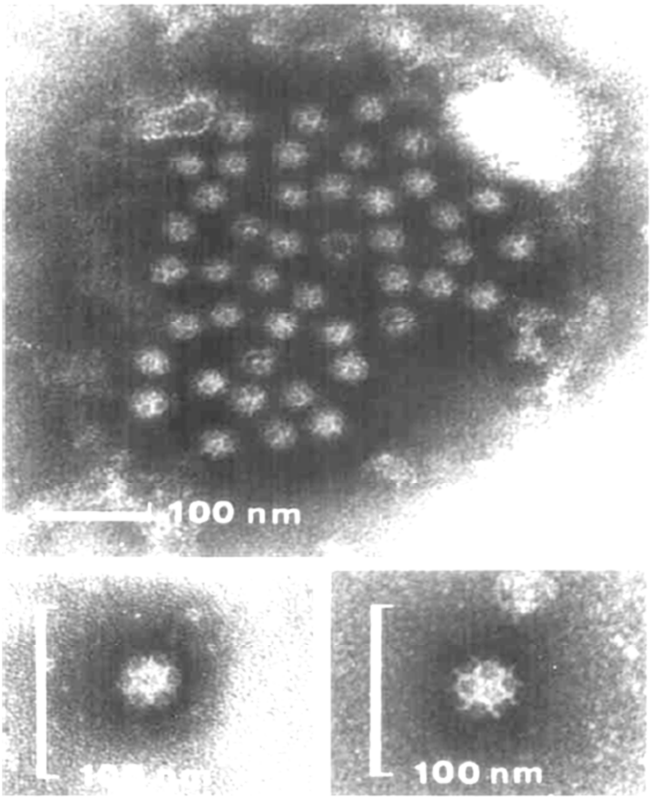

Figure 4. Electron microscopy of SaV viral particles isolated from fecal sample in Sapporo, Japan (Modified from Chiba *et al.*, 1979).

The inability to propagate SaV *in vitro* hampered the research progress. First attempts used different cell cultures, for example primary and secondary rhesus monkey kidney, primary human amnion, human embryonic kidney (HEK), among others; with no evidence of cytopathic effect (Madeley & Cosgrove, 1976; Chiba *et al.*, 1979). Also, trials inoculating human virions in mice, as animal models, did not succeed (Cubitt *et al.*, 1979).

The appearance of molecular techniques allowed the study of the virus in a more comprehensive way. A prototype classical human calicivirus, designated as “Sapporo strain”, was isolated, once again, from another outbreak in the well known infant home of Sapporo, in 1982 (Nakata *et al.*, 1985). Nucleotide sequence analysis of the RNA polymerase and the capsid protein regions of this 1982 Sapporo strain (Hu/SaV/Sapporo/1982/JPN) showed greater sequence similarities with the animal caliciviruses than with NoV or other SRSVs. This fact, together with the high similarity with the Manchester virus (MV), concluded with their inclusion in the genogroup III of the human calicivirus (Matson *et al.*, 1995; Numata *et al.*, 1997).

Caliciviruses from animals and humans were, by then, classified into 4 genera: *Vesivirus* (swine vesicular exanthema virus and feline calicivirus), *Lagovirus* (rabbit hemorrhagic disease virus and European brown hare syndrome virus), *Norwalk-like virus* (represented by NoV), and *Sapporo-like virus* (represented by SaV). Most of the viruses previously known as classical human caliciviruses and genogroup III human calicivirus (represented by the Sapporo strain) became members of the genus *Sapporo-like virus*, on the basis of genetic analysis (Numata *et al.*, 1997; Nakata *et al.*, 2000). The genus was divided into at least 3 genogroups, Sapporo strain genogroup, London/92 genogroup, and Parkville virus genogroup, on the basis of sequence differences (Berke *et al.*, 1997; Jiang et al.,

1997; Noel *et al.*, 1997). On 2002, the International Committee on the Taxonomy of Viruses (ICTV) established, into the family *Caliciviridae*, the genus *Sapovirus* and the species *Sapporo virus* (Mayo, 2002).

1.5.2. Taxonomy, molecular characterization and antigenicity

Genus *Sapovirus* is classified in the family *Caliciviridae*, which was established in 1974. Until then, all caliciviruses constituted a genus within the family *Picornaviridae*. Afterwards, the improvement of molecular techniques lead to the current characterization of five genus in the *Caliciviridae* family: *Sapovirus*, *Norovirus*, *Lagovirus*, *Vesivirus* and *Nebovirus* (Clarke *et al.*, 2012) (Figure 5); whereas five new genera have been proposed: *Bavovirus*, *Nacovirus*, *Recovirus*, *Valovirus*, and *Secalivirus* (Oka *et al.*, 2015).

The genome of the 1982 Sapporo prototype strain was completely characterized, showing more than 90% nucleotide sequence identity with the 1993 MV strain, the first SaV ever genotyped (Nakanishi *et al.*, 2011). Thus far (as of April 2018), 45 complete SaV genomes are available in GenBank (30 for humans and 15 from animals). The partial RNA-dependent RNA polymerase (RdRp), the major capsid protein (VP1) partial region or both of them, can be used to characterize SaV, as well as to investigate the similarities for epidemiological surveys. In contrast, the RdRp-VP1

junction region is too short for such sequence analysis. Therefore, VP1 sequences are widely used for genetic classification of SaV, as this region is more diverse than the RdRp (Farkas *et al.*, 2004) and the VP1 sequence correlates with virus antigenicity (Jiang *et al.*, 1997; Hansman *et al.*, 2007a). Moreover, the International Calicivirus Conference Committee proposed that at least the entire VP1 region sequence is necessary to designate new genogroups and genotypes. In accordance with this, ICTV latest report (2011) recognizes five SaV genogroups, from GI to GV. Human SaV belong in genogroups GI, GII, GIV and GV, with a total of 16 genotypes (GI.1–7, GII.1–7, GIV.1 and GV.1) plus an additional proposed GII.8 genotype; and porcine SaV is classified into GIII (Clarke *et al.*, 2012; Liu *et al.*, 2016) (Figure 6).

Phylogenetic analysis using complete capsid sequences of human and animal strains have recently revealed 19 different SaV genogroups, adding fourteen (from GVI to GXIX) to the actual classification, and establishing SaV as high diverse taxon (Scheuer *et al.*, 2013; Oka *et al.*, 2016; Yinda *et al.*, 2017). These studies used the pairwise distances of the capsid amino acid (aa) sequences in order to easily convert the distance to percent identity, using a genogroup cutoff of 0.40 (60% aa identity), which agrees with the actual published classification parameters for SaV and NoV (aa identity of 37-50% for intergenogroup, and 71-84% for intragenogroup). Most of the newly proposed genogroups were specific to a single host species; however, according to this new scheme, human GI would

infect also chimpanzees, human GII would be present in rats as well, and human GV would consist of porcine and sea lion strains too. Apart from the previously mentioned, the new genogroups would include more strains of porcine SaV (GVI, GVII, GVIII, GIX, GX, and GXI); and also mink (GXII), dog (GXIII), bat (GXIV, GXVI-XIX) and rat (GXV) strains. Unfortunately, the 2017 ICTV report does not include the corresponding calicivirus chapter yet in order to completely elucidate the official resolution of these proposals.

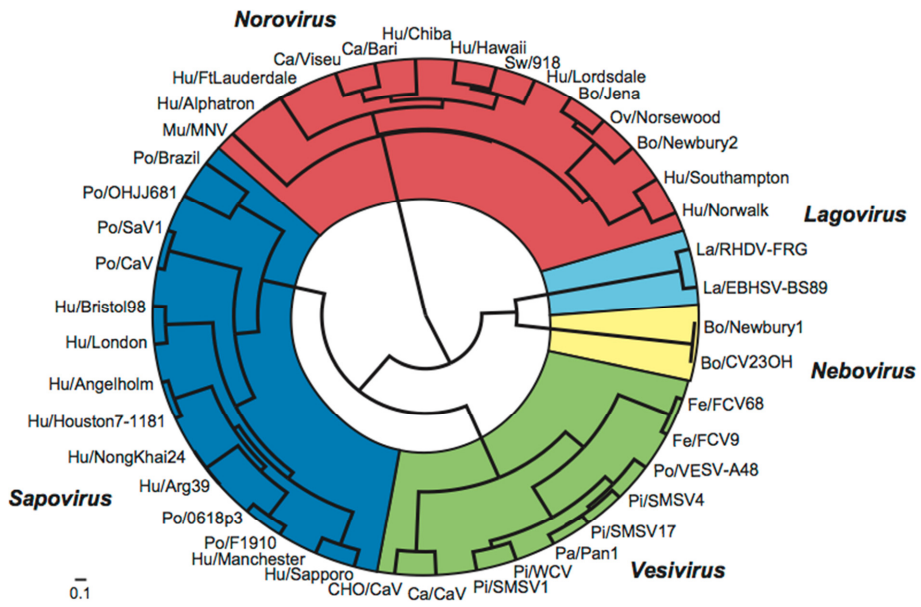

Figure 5. Phylogenetic relationships in the family *Caliciviridae*. Genera are defined by p-distance including all representative genogroups within a genus, employing amino acids of full length VP1 capsid sequences and neighbor-joining clustering method. The scale bar represents amino acid substitutions per site (Modified from ICTV, 2011).

Distinct antigenic diversity among SaV strains has been demonstrated by using clinical specimens, recombinant VP1 proteins, or virus-like particles (VLPs) (Jiang *et al.*, 1997; Hansman *et al.*, 2005; 2007a). Antigenicity differs through the human genogroups strains (GI, GII, GIV and GV), and is also distinct among different genotypes within GI and GII. Hyperimmune antiserum samples react strongly with homologous VLPs, but weakly when cross-reacted against heterologous VLPs in an antibody enzyme-linked immunosorbent assay (ELISA). Studies also demonstrated that at least two different epitopes are located on the capsid protein of human SaV in the four human genogroups (Kitamoto *et al.*, 2012). These experimental results also support VP1 as the determinant region for SaV antigenicity (Hansman *et al.*, 2007a). The antigenicity differences between human and animal SaV have not been determined yet.

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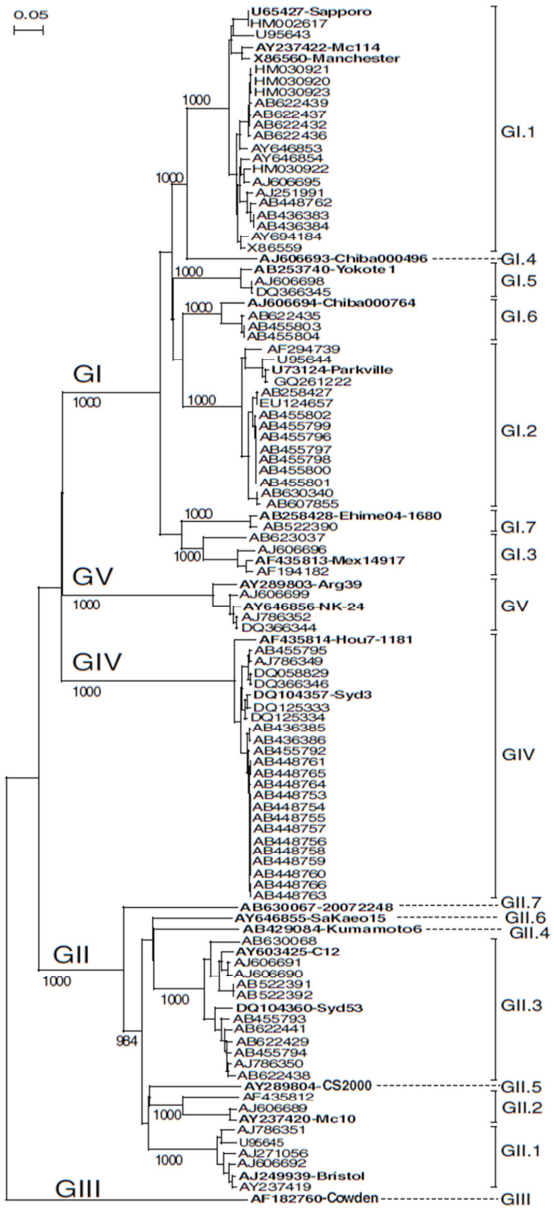

Figure 6. Phylogenetic tree constructed using the entire VP1 nucleotide sequences of 107 SaV strains, with the accession numbers and genotypes indicated. Representative strains of each genotype are in boldface. The scale represents nucleotide substitutions per site (Oka *et al.*, 2012).

1.5.3. Viral structure and physicochemical stability

Viral particles are small, of approximately 30 to 38 nm in diameter and a density of 1.36 to 1.41 g/cm^3. SaV are non-enveloped viruses with 180 molecules of VP1 as capsid (same as NoV), presenting an icosahedral morphology with cup-shaped depressions on their surface when observed with EM, which is the typical calicivirus morphology (Madeley, 1979).

A major obstacle to the study of SaV physicochemical stability is the lack of a routine cell culture system. Also, there are no assays for a direct evaluation of the inactivation efficiency. This problem is partially overcome by using surrogates, but an ideal one should be associated only with acute gastroenteritis and have a similar environmental resistance. Murine norovirus and feline calicivirus have been commonly used, but infections caused by these show different clinical manifestations compared to SaV. The cell culture-adapted porcine sapovirus (PoSaV) Cowden strain belongs to the same genus and the wild-type causes gastroenteritis in gnotobiotic pigs. Therefore, PoSaV is an alternative enteropathogenic calicivirus resembling human SaV (Chiba *et al.*, 2000; Green *et al.*, 2001).

Studies suggest a wide resistance to heat, pH and environmental conditions. Inactivation by heating after incubation at 56 °C for 30 min and 2 h, resulted in a reduction of infectious PoSaV in 2.3 and 4.0 $\log_{10}$ $TCID_{50}$, respectively. PoSaV became undetectable by cell culture infectivity assay after ethanol treatment (60% and

70%, vol/vol) at room temperature for 30 s. The stability against sodium hypochlorite was tested using different concentrations (2.5, 10, 50, 100 and 200 mg/L) at room temperature for 30 min, and the RNA became undetectable by reverse transcription (RT) real-time PCR (qPCR) only after 200 mg/L concentrations, but PoSaV had already lost infectivity at the lowest concentrations (2.5 mg/L) of sodium hypochlorite. PoSaV were found stable from pH 3.0 to 8.0 for 1 h, with a reduction of the infective dose of <1.0 $\log_{10}$ ($TCID_{50}$) at pH 3.0 compared to the value for pH 4.0 to 8.0. Finally, PoSaV significantly attached to lettuce leaves when at capsid isoelectric point (pH 5.0), and viral particles remained infectious on lettuce after 1 week of storage at 4 °C (Wang *et al.*, 2012).

1.5.4. Genomic organization and recombination

SaV genome is a linear +ssRNA with an approximately length of 7.5 kb, covalently linked to a virus genome-linked protein (VPg) at 5´-end and a polyadenylated tail at 3´-end. It has two open reading frames (ORFs) of which ORF1 encodes a large polyprotein containing the non-structural proteins and the major capsid protein, whereas ORF2 is predicted to encode a minor structural protein (Green, 2007). A third ORF (ORF3) has been supposed in several human SaV (Schuffenecker *et al.*, 2001; Farkas *et al.*, 2004), however, its function remains unknown (Figure 7). Other genera of family *Caliciviridae* present a similar genomic organization, but *Norovirus* differs as the

non-structural proteins are encoded into the ORF1, and the VP1 and minor structural protein (VP2) are located into ORF2 and ORF3, respectively (Green, 2007).

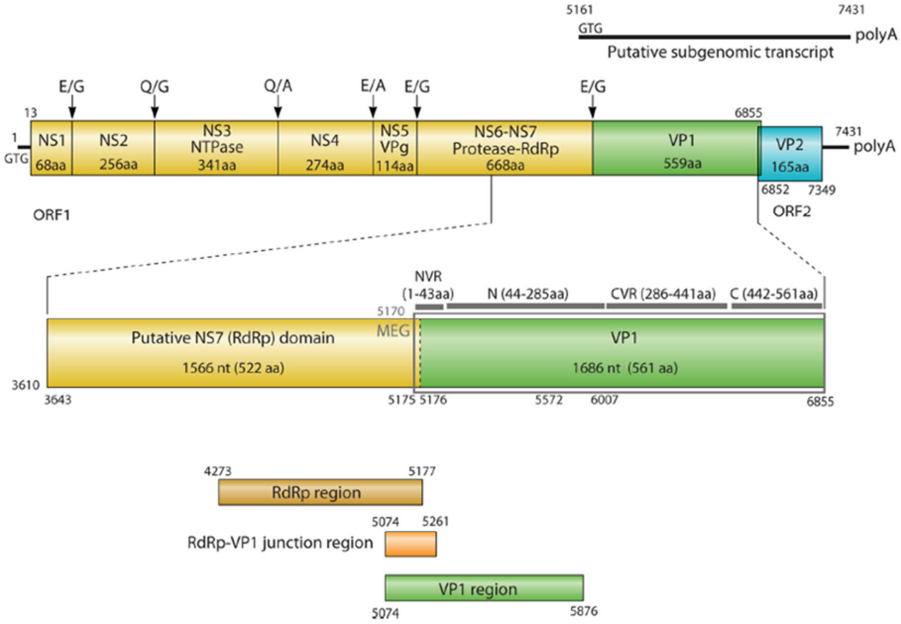

Figure 7. SaV genomic organization based on the GI.1 Manchester strain (GenBank-X86560). Diagram including the putative subgenomic transcript, two common ORFs, the predicted viral non-structural proteins (NS1 to NS6-NS7), and structural proteins VP1 and VP2. Also shows an overview of the RT-PCR target regions (RdRp, RdRp-VP1 junction, and VP1 regions), and the proposed subdomains in the VP1 subgenomic transcript (Modified from Oka *et al.*, 2015).

ORF1 polyprotein expresses non-structural proteins (NS) and the VP1, which are all processed by a virus protease. Some of the NS biological functions remain unclear, but NS3 is expected to act as a

nucleoside-triphosphatase (NTPase) and NS5 as VPg, based on their aa conserved motifs (Oka *et al.*, 2005). VPg is linked to the 5´-end tail of the SaV genome, playing an important role on the replication, transcription and translation of the virus. On the other hand, NS6 and NS7 appear to have proteolytic and polymerase functions, respectively (Green, 2007).

While the VP2 protein has not been identified in SaV virions yet, VP1 is known to be the major component of the viral capsid. It is a 60 kDa protein which production could happen either by its cleavage from the ORF1 polyprotein, or by its translation from a subgenomic RNA. The expression of VP1 in insect or mammalian cells result in the assembling of VLPs (Hansman *et al.*, 2008; Oka *et al.*, 2009), which are morphologically and antigenically indistinguishable from those of the native virions found in clinical samples. SaV VP1 can be separated into several subdomains, some of them presenting strictly conserved aa motifs among caliciviruses (Chen *et al.*, 2004).

Recombinant or chimeric strains refer to those classified based on the VP1 region, but with no concordance with the non-structural RdRp region. This inconsistent grouping has been detected both at genotype and genogroup level, being the most common found in GIV sequences (VP1 region) clustered with GII strains when analyzing the RdRp region. More data are needed spanning the RdRp region, as nowadays is less trustworthy due to the amount of sequences compared to the VP1 region. It has been suggested that, for a better

understanding of recombinant strains and for a reliable classification scheme for SaV, it would be critical to amplify a single PCR amplicon covering both RdRp-VP1 encoding regions (Oka *et al.*, 2015).

1.5.5. Replication and cell culture

Viral replication starts with the attachment of the viruses to the surface of enterocyte cells of the host through specific receptors. These first interactions are not yet well known, but the recognition of the receptor relies to be essential for the infective cycle of SaV, followed by the entry of virions through endocytosis. Once in the cytoplasm, VPg is removed from the 5´-end viral genome, which is then translated into an ORF1 polyprotein to yield the viral proteins. As for other (+)ssRNA viruses, the viral genome is infectious when introduced, so the RdRp uses the (+)RNA for the synthesis of a complementary strand with negative sense, resulting in dsRNA (Black *et al.*, 1978). When separated, the (-)RNA strand will be again replicated or transcribed providing new viral (+)ssRNA genomes or mRNA, respectively. Those mRNA copies will continue coding the translation of structural proteins VP1 and VP2, and finally will autoassemble generating viral particles with the (+)ssRNA genome copy within, releasing by cell lysis. The absence of corrector activity in the viral polymerase produces an error rate relatively high during transcription. This is the cause why SaV, as other RNA viruses, present high genetic diversity.

The establishment of an efficient *in vitro* cell culture system for human SaV remains challenging. All attempts until date have not succeeded, except for one reported in green monkey kidney cells (Kjeldsberg, 1977) and another in primary HEK cells in the presence of trypsin and actinomycin D (Cubitt & Barrett, 1984); which were reported during the last century but never confirmed. This absence has also hampered the development of typical immunological and serological techniques, because of the scarcity of antigens needed to produce antibodies. Currently, cell culture systems have been established only for PoSaV Cowden strain (best human SaV surrogate) using the porcine kidney cell lines LLC-PK1 (Parwani *et al.*, 1991). It was found that bile acids in intestinal content play a critical role in virus entry, allowing escaping from endosomes into the cytoplasm for PoSaV replication (Chang *et al.*, 2004), In fact, PoSaV replicates primarily in the proximal intestinal tract (duodenum and jejunum) where bile acid concentrations are high (Flynn & Saif, 1988). Bile acids also promote hepatitis B and C virus replication but inhibits RV multiplication (Shivanna *et al.*, 2014). This requirement is a rare phenomenon and implies important biological interactions in the intestine. However, molecular studies on PoSaV remain difficult due to slow virus replication rates and the low viral yield obtained from reverse genetics systems (Oka *et al.*, 2018).

1.5.6. Viral transmission and zoonosis

The main transmission of SaV is through the fecal-oral route, which might occur both directly and indirectly. The direct via includes person-to-person contact through vomit aerosols or feces containing SaV (from symptomatic and asymptomatic individuals); while the indirect pathway involves food, drinking and recreational water, surfaces or fomites, previously contaminated by feces or vomits from the primary dissemination (Gallimore *et al.*, 2005; Yoshida *et al.*, 2009; Iizuka *et al.*, 2010; Rasanen *et al.*, 2010; Kobayashi *et al.*, 2012) (Figure 8).

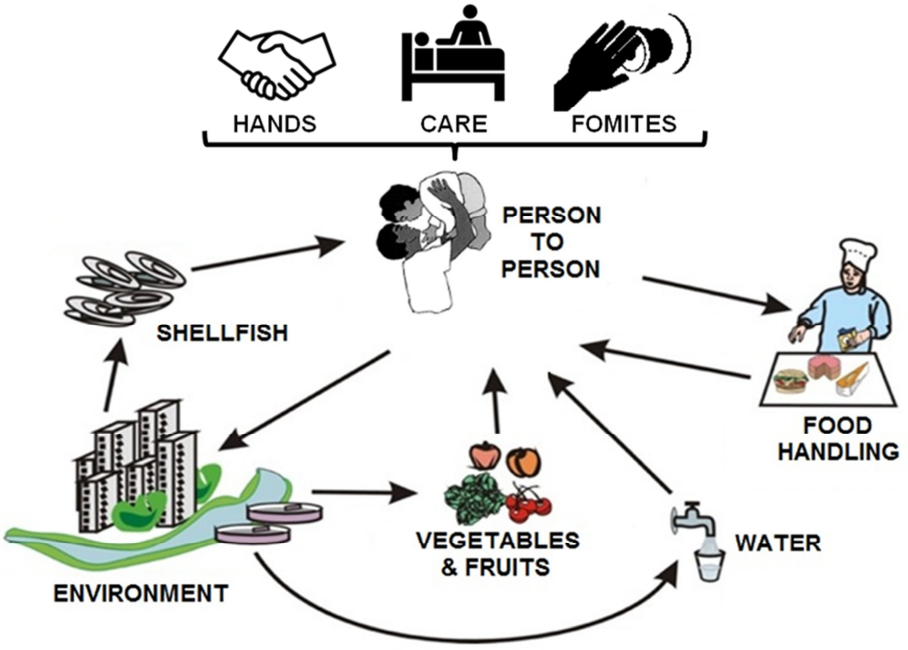

Figure 8. Scheme of SaV transmission through the fecal-oral route

The ratio relating the viral shedding load (10^5-10^{11} viral particles per gram of stool) with the viral dose required to cause an infection (i.e., 1,000 to 2,500 genomic copies) makes SaV a pathogen with a high transmission capacity (Teunis *et al.*, 2008; Atmar *et al.*, 2014). Also, their shedding period extends for more than 3 weeks, increasing the dissemination risks even after the disappearance of the symptoms (Iwakiri *et al.*, 2009). Direct transmission occurs in a short period of time, as the viruses leave the body of an infected person and can rapidly enter into another one. However, the secondary routes take advantage of their high stability in the environment, related with their great resistance to physicochemical agents, which increases the ability of SaV to infect people after long periods out of the body (Duizer *et al.*, 2004; Otter *et al.*, 2011; Wang *et al.*, 2012).

There are no evidences on SaV of any genetic host susceptibility or resistance to infection. Also, *in vitro* data have not shown evidences of SaV binding to HBGAs, contrary to NoV (Shirato-Horikoshi *et al.*, 2007), and therefore it has not been associated any HBGA phenotype with a susceptibility to the disease (Bucardo *et al.*, 2012). Re-infection is possible due to the absence of a long-term immunity, and it can be caused by the same or different SaV strains; which also makes difficult the development of efficient vaccines.

The emergence of zoonotic strains of human pathogens is experiencing a significant increment as it is influenced by the

frequency of contact between the animal reservoirs and human population (Parrish *et al.*, 2008). Until date, no clear discovery has been made on the existence of a SaV zoonosis affecting humans, but two studies appear to have discovered what could be the first evidences of interspecies cross-transmissions. On one hand, Mombo *et al.* (2014) reported the complete genome of two SaV strains identified in two fecal samples of chimpanzees living in a sanctuary in the Republic of Congo, in close contact with humans. The animals displayed no gastrointestinal symptoms and SaV genomes were 100% identical, both clustering within the human GI clade, and also presented 99.7% identity to the GI human SaV Plymouth isolate, suggesting recent cross-species barrier transmission of these viruses from humans to chimpanzees (Mombo *et al.*, 2014). On the other hand, a SaV strain was obtained from multiple tissues of a rat in New York City, including intestine, liver, salivary gland, and spleen, clustering within a monophyletic clade formed by SaV GII in the VP1 aa sequence. However, the pairwise nucleotide distances between the rodent strain and SaV GII genotypes indicated an intermediate taxonomic position, remaining unclear whether the rodent strain should be included into a new GII genotype or whether it should be a novel genogroup (Firth *et al.*, 2014).

1.5.7. Clinical symptoms and immunity

Incubation mean period obtained from SaV epidemiological data has been established in a range between 24 and 96 hours (Humphrey *et al.*, 1984; Noel *et al.*, 1997; Iizuka *et al.*, 2010; Yamashita *et al.*, 2010). Major clinical symptoms associated to infections caused by SaV are diarrhea and vomiting, but also include nausea, abdominal cramps, chills, headache, body malaise, and rarely fever. Feces do not usually present any blood or mucus. Symptoms normally disappear after 12-72 hours, and diarrhea resolves within 1 week (Chiba *et al.*, 1979; Humphrey *et al.*, 1984; Noel *et al.*, 1997; Mikula *et al.*, 2010; Lee *et al.*, 2012; Kobayashi *et al.*, 2012); even though, in some cases, their duration might last longer (over a week up to 20 days), mainly in infants and children, or in risk groups such elders or immunocompromised individuals susceptible to more serious clinical complications (Nakata *et al.*, 1985; Sakai *et al.*, 2001; Wu *et al.*, 2008). In infants and children, and contrary to adults, vomiting seems to occur more frequently than diarrhea.

In general, SaV gastroenteritis presents milder severity than RV and NoV (Pang *et al.*, 2000; Sakai *et al.*, 2001; Rockx *et al.*, 2002), and is usually a self-limiting disease with a recovery time of a couple of days. Some patients might present more severe symptoms, being estimated in less than 10 % the cases which required hospitalization due to dehydration and loss of electrolytes (Suzuki *et al.*, 1979; Phan *et al.*, 2004; Zintz *et al.*, 2005; Jin *et al.*, 2009; Medici *et al.*, 2012), as

already mentioned; and mortality episodes has been reported (Lee *et al.*, 2012).

Around 30 % of SaV infections are subclinical (asymptomatic) facilitating the transmission of the viruses to other human beings and making the source tracking difficult. Studies have revealed that SaV concentrations shed in feces of asymptomatic patients are comparable to those presenting gastroenteritis symptoms (Kobayashi *et al.*, 2012).

The serological responses to SaV infections using purified virus from feces or VLPs in IEM, ELISA, or radioimmunoassay (RIA) studies demonstrated that infection with human SaV commonly occur during early childhood, as the seroprevalence rates increase with age, showing high levels in children and remaining high in adults (Chiba *et al.*, 1979; Suzuki *et al.*, 1979; Cubitt *et al.*, 1981; Nakata *et al.*, 1985; 1988; Farkas *et al.*, 2006). The presence of SaV antibodies in serum has been associated with reduced frequencies of infection and illness, although protective immune mechanisms to SaV infection remain to be clarified. Affected individuals are thought to develop a short duration immune answer after the infection that offers a partial protection against posterior infections (Rockx *et al.*, 2002). Nevertheless, continuous infections with different SaV genogroups and genotypes were recently reported (Harada *et al.*, 2012).

1.5.8. Epidemiology, prevalence and environmental presence

SaV have been detected in more than 30 of countries worldwide, being part of more than 150 research studies and ranking second to fourth as the major viral pathogen when attending to sporadic gastroenteritis patients (Oka et al., 2015). The estimated prevalence ranges from 2.2 to 12.7 %, according to the results obtained using different methods (EM, ELISA, and PCR), but it reaches higher percentages in infants and children under five years old (Díez-Valcarce *et al.*, 2018). Nevertheless, SaV incidence in sporadic cases of acute gastroenteritis is not yet well documented and probably highly underestimated in many parts of the globe. This fact is caused by both a lack of routine clinical diagnosis (e.g., in Spain for RV and AdV, using ELISA), affecting prevalence studies, and a mild and self-limited disease with no requirement of hospitalization for most of the patients (Oka *et al.*, 2015).

As for many other enteric viruses, SaV incidence is higher during the cold and rainy seasons (Phan *et al.*, 2004; Johnsen *et al.*, 2009; Dey *et al.*, 2012), but sporadic cases have also been reported among warmer and drier months of the year (Pang *et al.*, 2000). On the other hand, SaV outbreaks do occur throughout the entire year in people from all ages and are estimated to cause 1.3 to 8.0 % of the gastroenteritis outbreaks. Outbreaks have been largely documented in several locations such as infant homes (Chiba *et al.*, 1979),

rehabilitation centers (Mikula *et al.*, 2010), residences for the elderly (Cubitt *et al.*, 1981; Lee *et al.*, 2012), kindergartens (Hansman *et al.*, 2007b), schools (Miyoshi *et al.*, 2010; Hergens *et al.*, 2017), colleges (Wu *et al.*, 2008), health care units and hospitals (Johansson *et al.*, 2005; Ishida *et al.*, 2008), restaurants, recreation and wedding halls (Nakagawa-Okamoto *et al.*, 2009; Yamashita *et al.*, 2010; Oka *et al.*, 2017), and even military bases (Neo *et al.*, 2017). To date, the largest recorded SaV outbreak was documented in Japan, with 665 patients apparently affected after the consumption of food from box lunches contaminated by infected handlers (Kobayashi *et al.*, 2012). Acute gastroenteritis outbreaks involving coinfections of SaV with other enteric viruses, such as NoV, RV, AsV or AdV, have also been reported (Nakata *et al.*, 2000; Lyman *et al.*, 2009; Iizuka *et al.*, 2010; Rasanen *et al.*, 2010; Iritani *et al.*, 2014). Some of these reported SaV gastroenteritis outbreaks have been associated with the consumption of shellfish, such as clams and oysters (Nakagawa-Okamoto *et al.*, 2009; Iizuka *et al.*, 2010; Wang *et al.*, 2015).

It has been observed that human genogroups and genotypes present differences in prevalence and distribution around the globe; and it is yet to be determined if they also have different transmission routes. Genetic characterization has been used as a helpful tool to determine the SaV strains circulating among the population, and the evolution of those in time in a geographical area (Svraka *et al.*, 2010; Harada *et al.*, 2012). It is also interesting to record the appearance of

recombinant strains, which could have altered their virulence, possibly leading to an increased disease burden (Cannon *et al.*, 2017). Genogroups GI and GII are the most commonly SaV observed worldwide in recent years. Among them, genotype GII.3 was predominant in Japan during the first years of the 21st century, but gradually disappeared after 2008 (Harada *et al.*, 2009; Harada *et al.*, 2012). In Europe and America, GI.I, GI.2 and GII.1 are the main detected genotypes (Gallimore *et al.*, 2006; Svraka *et al.*, 2010; Díez-Valcarce *et al.*, 2018). Genogroup GIV was first discovered in children from Pakistan in 1992 (Phan *et al.*, 2004), and although viruses belonging to this genogroup are rarely reported, they have been also detected recently in Japan, USA and Europe (Oka *et al.*, 2015). In a recent study about the genetic diversity of SaV across four American countries, GIV strains showed as the second most common genogroup (Díez-Valcarce *et al.*, 2018). The other human genogroup, GV, has not been widely detected since it was reported in 1995 in Argentina for the first time (Farkas *et al.*, 2004).

When studying the epidemiology and prevalence of SaV in a population, it is important to take into account the presence of the virus in the environment, as it is in river water and wastewater, where human feces from infected individuals get discharged, reaching the coast at some point, and becoming potentially accumulated by shellfish. Similar SaV strains have been detected and genetically characterized (based on partial genomes) in human

clinical and environmental samples, such as river water (Kitajima *et al.*, 2010; Sano *et al.*, 2011), wastewater (Kitajima *et al.*, 2011; Varela *et al.*, 2018), and also in clams (Iizuka *et al.*, 2013) and oysters (Ueki *et al.*, 2010), even though studies on shellfish are scarce. This last cited study (Ueki et al., 2010) even detected similar SaV strains from patients, wastewater and oysters collected in the same months at geographically related areas.

The presence of SaV in the environment normally increase during cold months, which is clearly correlated with the number of people suffering from acute gastroenteritis caused by SaV, and therefore, shedding viruses. Studies conducted about quantification levels of SaV in wastewater are scarce. In those, the maximum values of concentrations in wastewater ranged between 10^4 and 10^8 genome copies GC/L (Haramoto *et al.*, 2008; Fioretti *et al.*, 2016; Varela *et al.*, 2018). Untreated wastewater reach coastal areas out of inefficient WWTPs or due to the inexistence of treatment facilities, and shellfish filtrating those waters accumulate on their body loads that can reach approximately 10^4 GC/g of digestive tissue in species of oysters, cockles and clams (Benabbes *et al.*, 2013).

1.5.9. Main detection techniques

Until the appearance of molecular techniques, enteric viruses were identified mainly using EM. SaV viral particles are distinguishable from other gastroenteritis pathogens like NoV, RV,

AsV or AdV due to the characteristic morphology of “star of David” under EM (Madeley, 1979), but the several intrinsic limitations of this technique made to be practically abandoned currently for laboratory or clinical diagnosis.

Afterwards, an ELISA was developed for the detection of human SaV antigens and, therefore, for the study of the virus in clinical samples (Jiang *et al.*, 1997), but sensitivity is not high enough to be used in low virus concentration scenarios when analyzing environmental and food samples. Furthermore, there is a current shortage of availability on commercial kits for this technique and it is quite difficult to detect antigenically diverse strains. The development of a broadly reactive ELISA could be possible, as a common epitope is expected to exist in all human genogroups (Kitamono *et al.*, 2012), but for now it depends on the combination of a panel of specific antisera or reactive monoclonal antibodies.

Methodological advances in molecular biology lead to the development of RT-PCR as the most employed and routine method for SaV research, being major the use of RT-qPCR due to its capability to quantify copies of genome. Multiplex RT-PCR or PCR assays, whose products get differentiated by agarose gel electrophoresis, and microsphere based fluorescent PCR product detection assay have also been reported for the detection of human SaV, as well as for other enteric viruses (Oka *et al.*, 2015). The nucleic acid detection methods brought more specificity, sensitivity and broad reactivity

than EM or ELISA. These techniques have been helpful to clarify during the last years the transmission of SaV throughout foods and the environment, as well as to detect SaV from clinical specimens in routine analysis. Primers designed for the detection of SaV target the partial RdRp, the RdRp-VP1 junction or partial VP1 regions. Since 2006, most research groups have used the same RT-qPCR protocol for the detection of human SaV which is able to detect viruses from all 4 genogroups in one assay, targeting the RdRp-VP1 junction (Oka *et al.*, 2006).

RT-PCR protocols targeting the partial VP1 region are sometimes preferred because the amplification products can be sequenced for reliable genotyping (Kitajima *et al.*, 2010). However, the success rates reported by genetic characterization studies for SaV range from 43 to 100 %, indicating that the current RT nested-PCR assays do not detect all circulating strains or that they are not efficient amplifying the low level of viral RNA present in some types of samples, such as shellfish (Murray *et al.*, 2016; Díez-Valcarce *et al.*, 2018).

Routine diagnosis analyses normally do not employ complete genomic characterization of the SaV genome due to the laborious work required. With the actual methodologies it is needed to amplify a long 2-2.5 kb region, to determine the complete VP1 using forward primers and a reverse primer hybridized to the 3´-end poly(A) tail. Apart from that, the amplification of the 5´-end would consist of a 5-

5.5kb fragment starting at the beginning of the genome and reaching the VP1 upstream region, for which there exists a lack of universal primers (Oka *et al.*, 2015).

New research technologies have been developed during the recent years, bringing solutions to the limitations of RT-PCR and RT-qPCR. On one hand, Digital RT-PCR (RT-dPCR) may solve some of these limitations, being less sensitive to inhibitory molecules presented in the sample, not needing a standard curve to quantify, and obtaining absolute quantification results. RT-dPCR divides each reaction mix across thousands of individual PCR reactions containing zero, one or more copies of the target sequence, and quantification is calculated using binomial Poisson statistics after endpoint (Pinheiro *et al.*, 2012; Rački *et al.*, 2014). Currently there is not a specific RT-dPCR protocol to detect only SaV, and just a few methodologies have been developed to study them, simultaneously with other enteric viruses (Coudray-Meunier *et al.*, 2016).

On the other hand, another disadvantage of using conventional molecular methods (e.g., PCR and qPCR) may be that they only detect the agents we are looking for, and it is normally not possible to obtain phylogenetic information. Metagenomic approaches enable the detection of several pathogens at the same time and it is useful in studying non-cultivable organisms, like SaV. Next generation sequencing (NGS) techniques have been already used to determine SaV genome sequences from humans (Zhou *et al.*,

2016; Joensen *et al.*, 2017), as well as to study SaV present in environmental samples like wastewater (Ng *et al.*, 2012) and sludge (Bibby & Peccia, 2013), and also in animals such as sea lion (Li *et al.*, 2011a) and dogs (Li *et al.*, 2011b). At present, however, this approach is too expensive and time-consuming, but as NGS becomes cheaper, their huge potential could be implemented for routine sampling diagnosis by direct sequencing of viral pathogens, such as SaV (Joensen *et al.*, 2017; Díez-Valcarce *et al.*, 2018).

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2. JUSTIFICATION & OBJECTIVES

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2. JUSTIFICATION & OBJECTIVES

The general objective of this doctoral thesis was the acquirement of a broader knowledge concerning sapovirus (SaV), one of the most important emerging enteric viruses for which an enormous lack of information exists. From an epidemiological point of view, hepatitis A virus and norovirus are the most noticeable enteric viruses transmitted through the fecal-oral route, leaving other important viral pathogens unfairly underrated in their worldwide importance. Until the execution of the studies conducted in this thesis, only anecdotic SaV detection had been reported in clinical samples from Spain (Sala *et al.*, 2014; Torner *et al.*, 2016; Gonzales-Gustavson *et al.*, 2017), and they had not been detected in harvested shellfish out of Japan. All this together, brings novelty to the analysis developed. Besides, the advances in molecular methodologies made possible the design of new tools for the study of enteric viruses, such as the digital PCR. The limitations of the largely employed qPCR can be now overtaken using the protocol detailed in this thesis for the detection and quantification of sapovirus in the most commonly studied sample matrixes.

The present doctoral thesis is divided in the next main objectives:

- To evaluate the presence and genetic diversity of human sapovirus in several commercialized species of shellfish grown and harvested in different Galician *rías*.
- To evaluate the incidence of human sapovirus among the population affected by acute gastroenteritis and their role as an etiological agent in the geographical area of the city of A Coruña.
- To determine the molecular epidemiology of human sapovirus and their population dynamics within the clinical study, and to establish a possible epidemiological relation between the acute gastroenteritis episodes and the consumption of bivalve molluscs contaminated with this viral agent.
- To develop a new digital PCR methodology capable of perform accurate analysis on the most common sample matrixes, in order to improve the study of human sapovirus using the latest molecular tools available.

3. RESULTS

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3. RESULTS

3.1. Study of sapovirus in bivalve molluscs of Galicia

3.1.1. Article 1. *Prevalence and genetic diversity of human sapoviruses in shellfish from commercial production areas in Galicia, Spain*

http://aem.asm.org/content/82/4/1167

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3.1.2. Article 2. *Human sapovirus in mussels from Ría do Burgo, A Coruña (Spain)*

https://link.springer.com/article/10.1007/s12560-016-9242-8

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3.2. Presence of sapovirus in the Galician population

3.2.1. Article 3. *Human sapovirus among outpatients with acute gastroenteritis in Spain: a one year study*

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Human sapovirus among outpatients with acute gastroenteritis in Spain: a one year study

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Abstract

Objectives: Viral agents of human gastroenteritis affect people of all ages across the globe. Studies are mainly focused on other enteric viruses, leaving sapovirus (SaV) underestimated as an important emerging human threat. The objective of the study was to determine the prevalence and genotypes of human SaV circulating among the Spanish population in the Galician region during one year.

Methods: This one year study analyzed clinical samples from hospital outpatients with acute gastroenteritis in Spain, with the aim of revealing the importance of human SaV as an emerging viral pathogen. A total of 2,667 stools were tested using RT-qPCR to detect and quantify SaV. RT-nested PCR and sequencing were used for further genotyping.

Results: Prevalence was 15.64 % (417/2,667), being slightly higher in 0-2 and 3-5 years old (19.5% [173/886] and 17.9% [35/195], respectively), and much lower in 13-18 (9.9%; 7/71). Positive samples were detected along all the year, with peaks of detection during autumn and late winter – spring months. Mean value for quantified samples was 6.5 x 10^5 SaV genome copies per gram of stool (GC/g) (range 2.4 x 10^3 - 6.6 x 10^{11} GC/g). Genetic characterization showed a predominance of GI, followed by GII and GIV.

Conclusions: Sapovirus were detected in all age-groups, especially in infants, children and elders. Detection of multiple genotypes suggests circulation of different strains without any clear tendency. Results obtained suggest SaV as the second major gastroenteritis agent after NoV in the region.

Keywords: Sapovirus; Gastroenteritis; Detection; Genotyping.

Introduction

Foodborne diseases have been, during the last years, the 8th major cause of human deaths, becoming 3rd in the African continent, and the cause of 9 % of mortalities in children worldwide, according to the Global Health Observatory [1]. Parasites, bacteria and viruses are the infectious agents causing thousands of deaths every year associated with these illnesses. Enteric viruses are among the major contributors, including a wide range of etiological agents as adenovirus (AdV), astrovirus, rotavirus (RT), aichivirus, sapovirus (SaV), hepatitis A and E virus, and norovirus (NoV). Most of them are considered foodborne viruses, but their transmission occurs not only via ingestion of infected food and water (irrigation of fruits and vegetables; and shellfish harvested in polluted waters), but also person-to-person (infected workers and handlers), contaminated environments and fomites. They are characterized by low infectious dose and high levels of viral excretion [2].

The genus *Sapovirus*, from the family *Caliciviridae*, has been recently included into the Contaminant Candidate List (CCL4) by the US Environmental Protection Agency as an emerging microbial agent producing mild gastrointestinal illness [3]. SaV are classified into five genogroups, four of which (I, II, IV, V) infect humans; even though the existence of several additional genogroups infecting different animals has been suggested. Their morphology correspond to a non-enveloped 30-38 nm diameter icosahedral virus, with single-stranded positive-sense RNA and a genome containing two or three open reading frames, depending on the genogroup [4].

Outbreaks happen throughout the year in all age-groups in different settings, such as children care centers, schools, colleges, hospitals, restaurants, hotels and cruises. SaV transmission occurs by the cited fecal-oral route and is extremely contagious, owing to a very low infectious dose, similar to NoV [5]. Incubation period lasts from less than a day until 4 days

[6]. Patients present acute diarrhea, with excretion of 10^5 to 10^{11} SaV genome copies per gram of stool (GC/g), and vomiting as major symptoms, but also abdominal pain, chills and headache have been reported. Illness is usually self-limiting with therapy just for dehydration and electrolyte imbalances, showing recovery in the first days, although some individuals may require hospitalization [7]. Shedding levels persist high even several weeks after (more than 10^5 GC/g) [8], and none or limited long-term immunity results from infection, meaning that one person may be repeatedly infected. Also, asymptomatic cases have been reported [9].

The main purpose of the study was to determine the presence of human SaV circulating among the Spanish population in the Galician region during one year, taking into account an age-group classification, as well as the drifts in time of the different genotypes.

Methods

Sampling

The study comprised stool samples from a total of 2,667 outpatients suffering from acute gastroenteritis treated at the Universitary Hospital of A Coruña city, in Spain; which serves to more than half million people. Samples were divided by age-groups as follows: 0-2 (n=886), 3-5 (n=195), 6-12 (n=244), 13-18 (n=71), 19-59 (n=653), ≥60 (n=597) and unspecified age (n=21). Sampling was carried out from July 2010 to June 2011, receiving between 31 and 112 stool samples weekly at the laboratory (58 on average). Coinfection analysis was performed with previously obtained data for RT, AdV and bacterial pathogens detected at the hospital, and for human NoV genogroups I and II at our laboratory [10].

Viral RNA extraction

Samples employed were previously processed and viral RNA extracted during the cited study [10]. Briefly, they were diluted 10 % (weight/volume) with supplemented PBS, shaken and

centrifuged for viral recovery. RNA extraction was carried out using NucleoSpin® RNA Virus kit (Macherey-Nagel, Germany) and adding known amounts of Mengovirus clone vMC_0 (MG) as extraction efficiency control. If needed, viral RNA was re-extracted from the original stool sample homogenate, stored in our facilities at -80 ºC.

RT-qPCR methodology

Detection and quantification methodologies were based on the principles established in the latest ISO procedures for enteric viruses [11, 12]. It was performed with reverse primer SaV1245R (5´-CCCTCCATYTCAAACACTA-3´), forward primer SaV124F (5´-GAYCASGCTCTCGCYACCTAC-3´) and TaqMan probe SaV124TP (5´-AM-CCCCTATRAACCA-MGB-NFQ-3´) under the following thermal conditions: reverse transcription at 50 °C for 20 minutes, denaturation at 95 °C for 15 minutes, continued with 45 amplification cycles with denaturation at 95 °C for 15 seconds, and annealing and elongation at 50 °C for 1 minute [13]. RT-qPCR was conducted using Platinum Quantitative RT-PCR ThermoScript One-Step System kit (Invitrogen, France) in a MX3000P QPCR System thermocycler (Stratagene, USA) in 96-well plates.

For all the samples, dilution 1:10 was also tested to reduce the effect of potential polymerase inhibitors present in the matrix, and for some samples dilution 1:100 was necessary as well. Negative control for each assay consisted in a well in the plate with nuclease free water and same RT-qPCR mix. Extraction efficiency control by MG consisted in the comparison between the threshold cycle value (C_t) of the MG positive control and the C_t for each analysed sample; the efficiency was considered valid if above 5 %. The presence of polymerase inhibitors and the RT-qPCR efficiency was calculated using external controls (EC) in each assay plate consisting in independent wells with 2.5 µL of sample and 2.5 µL SaV positive control. C_t values of the EC were compared with those of the positive control well,

considering the amplification valid if the efficiency was above 25 %. Control materials were obtained by cloning the 112 bp target fragment of the polymerase-capsid junction (nucleotide positions 5073–5184 of GI Sapporo strain full-length genome; accession number HM002617) into pGEM-T easy plasmid, transforming *Escherichia coli* DH5a competent cells, then purifying and quantifying using spectrophotometry (A260 nm). After this, standard curves were calculated using serial dilutions. Neither the extraction efficiency nor the RT-qPCR efficiency were used to modify the quantification values obtained in the samples. Limit of detection (LOD) was settled in 5.2 x 10^2 GC/g and limit of quantification (LOQ) in 2.2 x 10^3 GC/g.

Genetic analysis

Genotyping of the detected SaV strains were carried out using a RT nested-PCR protocol that amplifies a partial fragment of the capsid gene of human genogroups [14]. RT consisted in 5 µL of positive SaV RNA template in a final volume of 20 µL mix, using RevertAid Reverse Transcriptase kit (Thermo Scientific, USA) with reverse primers SV-R13 and SV R14 (1 µM) for 60 min at 42 °C. First-round PCR was conducted using 5 µL of cDNA in a final volume of 50 µL, with the same reverse primers and forward primers SaV124F, SaV1F and SaV5F (all 0.5 µM). Amplification conditions were: 94 °C for 2 minutes denaturation, then 40 cycles of 94 °C for 30 seconds denaturation, 50 °C for 30 seconds for primer annealing, 72 °C for 2 minutes for extension, and finally 72 °C for 10 minutes for late extension. Second-round PCR was performed for 45 cycles under similar conditions (extension 1 minute) with primers SV-R2 and 1245Rfwd (0.5 µM) and 5 µL from the first-round PCR product. Both amplifications were performed with Fermentas DreamTaq DNA Polymerase kit (ThermoScientific, USA). Nested-PCR products were visualized in 2 % agarose electrophoresis gels and amplicons of approximately 420 bp size were considered for purification and sent to be genotyped.

The obtained sequences were aligned and analysed with software packages Lasergene 7 (DNASTAR inc., Madison, USA) and MEGA version 6 [15]. Neighbour-Joining (NJ) phylogenetic dendrograms were constructed with software MEGA version 6 using the Kimura two-parameter model (bootstrap of 1,000 replicates). If some weeks of the study presented an unusual upturn of positives samples belonging to the same genotype, they were analysed using MegAlign (DNASTAR®, USA) in order to find out whether they could constitute possible outbreaks. SaV reference strains were retrieved from GenBank, and strains obtained in this study are also available at GenBank under accession numbers LT985022-LT985081, LT985914-LT985948, LT986236-LT986326.

Statistics

Statistical analysis was conducted with R Statistical Software version 3.4.3 (R-Project, Vienna, Austria). Analytical experimental data on detection frequencies were analysed through a chi-square test to determine if the SaV prevalence was dependent on the age. In the same way, quantification results were converted into a logarithmic format and tested with a one-way analysis of variance (ANOVA) to study any relationship between the SaV viral loads with the age-groups [16].

Results

Prevalence

Extraction and RT-qPCR efficiencies were classified as valid in all cases (>5 % and >25 %, respectively). Molecular assays were performed in the 2,667 stool samples and human SaV were detected in 417 (15.64 %) of them. Results obtained showed that human SaV were present in all age-groups, with a higher incidence in infants under 2 years old (173/886, 19.5 %) and in children 3-5 years old (35/195, 17.9 %). The rest of the age-groups rendered as

follows: 6-12 (29/244, 11.9 %), 13-18 (7/71, 9.9 %), 19-59 (79/653, 12.1 %) and ≥60 (90/597, 15.1 %). Statistical analysis with chi-squared test showed a significant correlation of SaV detection with age-group (p = 5.9 x 10^{-4}). Prevalence decreases from childhood to the adult years, and it rises again in elders above 60 years old (Figure 1).

Coinfection

From the 417 total SaV positive samples, 196 presented at least another gastroenteric microbial pathogen (Figure 2). SaV showed coinfection with NoV in 164 cases, of which 85 were with genogroup I, 71 with genogroup II and 8 with both genogroups. Of those 85 SaV-NoV GI detections, 6 samples were positive for *Campylobacter* spp., 3 samples for *Salmonella* spp., 2 samples for *Bacillus cereus*, and 1 sample presented *Pseudomonas aeruginosa* too. Among those other 71 SaV-NoV GII positive samples, bacterial pathogens *Staphylococcus aureus* and *Yersinia enterocolitica* were present in 1 patient each one, and *Salmonella* spp. in other 2 cases. On the other hand, some pediatric cases were also previously analysed at the hospital for RT and AdV (total of 125) and 3 coinfections were detected with SaV-RT, 2 with SaV-RT-AdV, and 1 with SaV-RT-NoV GII (Figure 2).

When analyzing these results taking into account the age-groups classification, it was observed that in 0-2 age-group occurred all the SaV-RT (including SaV-RT-AdV), as well as most of the detected coinfections with *Campylobacter* spp. and *Salmonella* spp. (Supplementary Figure S1A). Apart from that, the combined presence of SaV with NoV GI and *Bacillus cereus* was only detected in the 19-59 age-group, and other different coinfections with *Pseudomonas aeruginosa*, *Aeromonas* spp. or *E. coli* 0157:H7 occurred just in the ≥60 years old age-group (Supplementary Figures S1E and S1F).

With the previous and actual analysis gathered for single detections and coinfections, the possible etiology of the illness could be elucidated for 47.4 % of the examined gastroenteritis cases included in this study.

Seasonality

The highest number of SaV detections were not observed during the most cold and rainy months of the study, but in early Autumn and late Winter-Spring (Supplementary Figure S2). September, October and February showed important prevalence percentages (21.6, 20.4 and 21.1 %, respectively). All age-groups followed this pattern, although prevalence of ≥60 presented an increasing tendency since November (Supplementary Figure S2).

Quantification

A total of 129 positive samples ranged below the LOQ (2.2 x 10^3 GC/g). The other 288 cases rendered an overall mean value of 6.5 x 10^5 GC/g, with a minimum value of 2.4 x 10^3 GC/g and a maximum of 6.6 x 10^{11} GC/g (Figure 3). Variations among viral loads in the age-groups were significant when analysed by ANOVA ($p = 2.0 \times 10^{-4}$). Age-group between 0-2 presented the highest quantification, while elders ($\geqslant 60$ years old) the lowest. When comparing age-groups of infants and children with elders, the differences of quantification were 1.5 log units between 0-2 and ≥60 ($p = 1.4 \times 10^{-7}$), and 1.0 log unit between 3-5 and ≥60 (p = 0,037) (Figure 3).

Genetic characterization

Sequences suitable for genetic characterization were obtained for 186 positive samples. Of them, 126 corresponded to genogroup I, 55 to genogroup II and 5 to genogroup IV. Genogroup I sequences were classified into GI.1 (n=91), the most predominant genotype, GI.2 (n=32) and GI.3 (n=3). On the other side, genogroup II sequences were split into

genotypes GII.1 and GII.4 (n=27 and n=21, respectively), genotype GII.2 (n=1) and GII.5 (n=6). Finally, all genogroup IV sequences belonged to genotype GIV.1 (Figure 4).

The temporal distribution of the different genotypes showed a predominance of GI.1 during the first quarter of the study, approximately from July until the end of October; followed by a decrease in the overall detection during late Autumn – early Winter. Later, the general diversity increases with genotypes GI.2 and GII.1 detections, and the appearance of GII.4. At the end of the study, only GI.1 and GI.3 remained as dominant genotypes (Figure 5).

Although GI.3 sequences (n=3) were detected only in infants 0-2 and children 3-5, and genogroup IV cases only occurred during childhood periods (Table 1), no clear tendency was observed for the most prevalent genotypes, as GI.1, GI.2, GII.1 and GII.4 sequences are present in all groups.

Short-term upturns of certain genotypes were observed several times along the study. In order to analyze in depth these results that could point towards the existence of outbreaks, sequence similarity was determined for these groups by MegAlign. The first group consisted of 11 GI.1 sequences detected in mid September, which presented 100 % similarity. The second group was 26 GI.1 sequences during October, of which 16 samples presented 100 % identity. Next genotype peak was on March-April with 24 GI.1 sequences, and 12 presenting 100 % identity. Finally, 9 out of 11 GII.4 sequences were similar during June (Supplementary Figure S3). Apart from these, several other upturns happened with similarity percentages ranging mostly between 99.0 and 99.9 % (data not shown), and could indicate the circulation of also other genotypes. No epidemiological data could be obtained to confirm the linkage among cases or the existence of a common viral vehicle.

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Discussion

The present study provides novel data about the role of SaV in patients with an acute gastroenteritis diagnosis receiving health care in Spain. Taking together our results with these from a previous study [10], from the 2,667 cases, the etiology was established in 47.4 % of them, including both bacterial and viral agents.

Most of the SaV long-term studies worldwide show prevalence rates ranging from 2.2 to 12.7 % [5], and many are located, as this research, in developed countries like Japan, USA, or UK [17-19]. The present study shows slightly higher SaV prevalence (15.6 %), more in line with the 17 % positive rate found in Nicaragua [20].

Moreover, patients from 0-2 and 3-5 years old presented the highest positive rates, which is in accordance with the established notion of higher prevalence of this pathogen in children [19, 21]. Elder people of ≥60 presented 15.1 % prevalence, demonstrating that together, with the infants, they are considered to be the most vulnerable population against viral acute gastroenteritis [22].

Mixed infections in patients could lead to more severe gastrointestinal symptoms and, therefore, analyses of clinical cases for more than one viral agent are needed. In 164 samples (39.3 %) NoV and SaV were simultaneous detected, which is a much higher level than what can be found in the literature, where mixed infections with both caliciviruses do not usually exceed 10 % of cases [17-20]. NoV-SaV mixed infections in the present study could be explained by the elevated prevalence of SaV, compared to other surveys. Pediatric coinfections with RV are scarce [18, 19] and it only occurred in 6 cases of this study, 3 of which showed triple mixed infections: 2 with SaV-RT-AdV and 1 with SaV-RT-NoV.

Regarding seasonality, SaV were detected along the entire period. Their incidence was higher in September, October and February; and its prevalence was consistently high during spring

months, in line with other studies [23]. It is normally accepted that SaV are mostly detected in the cold and rainy seasons [24, 25], which is not in concordance with our results. Observing the results obtained previously for these samples [10], the highest prevalence rates of NoV cases occurred between November and January (when the SaV prevalence was at its lowest), and moreover, NoV prevalence rates decreased during the mentioned SaV high peaks of abundance (data not shown).

Mean values of quantified samples was 6.5 x 10^5 GC/g of stool, which are in accordance with most of the studies providing information about shedding levels [8]. These levels gradually decrease after onset of illness and therefore, based on the detection of mixed infection, some low viral load quantification cases (also under LOQ) could be, hypothetically, a combination of a late SaV – early coinfection with other pathogens, which could cause the severe symptoms of acute gastroenteritis with hospital care requirements.

Genetic analysis is crucial to elucidate the current circulating SaV in the population, regionally and worldwide. Investigations have revealed that GI and GII are the most common SaV genogroups [26]. Genotype I.1 is the most common genotype in the globe, and GI.2 has been increasingly detected in many European countries [27], and also in northern Africa [28]. Results from this research support those from previous studies, as GI.1 and GI.2 were the two most abundant SaV genotypes (91 and 32 cases, respectively), followed by GII.1. Regarding the genotype GII.4, it was detected in this study in 21 samples. Although it was predominant in Japan during the last decade (2003–2009) [29], it does not represent a major circulating variant among the European region. On the other hand, GIV.1 was a genotype normally detected at high rates in developed countries around 2007 [5]. Environmental studies conducted in Spain obtained findings that mirror what was observed in this study, showing a rich variety of sequences circulating, especially genotypes GI.1 and GI.2 [14, 30, 31].

Unfortunately, no epidemiological data were available for a more complete understanding of the genetically analyzed upturns, which could have been considered as hypothetical outbreaks. Surveillance systems in hospitals and health care centers are mostly limited to detect bacterial pathogens. Since many of the enteric viruses produce mild and often uninvestigated infections, their recognition as possible critical human pathogens has been underestimated. Most of them are difficult to study and have symptoms similar to those caused by bacterial pathogens. This study would help positioning human SaV, at least, as one of the foremost cause of non-bacterial acute gastroenteritis agents in Spain, especially in some age-groups.

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Transparency declaration

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Table 1.- Genetic distribution of SaV in the different age-groups (years old).

Genogroup	Genotype	0-2	3-5	6-12	13-18	19-59	≥60	Undet.	TOTAL
GI	GI.1	41	8	8	1	14	19		91
	GI.2	11	5	4		5	5	2	32
	GI.3	1	2						3
GII	GII.1	17	1	1		6	2		27
	GII.2		0			1			1
	GII.4	7	1	1	1	5	6		21
	GII.5	2	1			1	2		6
GIV	GIV.1				1	2	2		5
TOTAL		79	18	14	3	34	36	2	186

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Figure legends

Figure 1. SaV negative (light grey) and positive samples (black), as well as human SaV prevalence within the different age-groups.

Figure 2. Diagram showing the number of SaV coinfections with other viral and bacteriological agents. Data for other pathogens obtained from previous study [10].

Figure 3. Boxplot showing logarithmic SaV quantifications for each age-group.

Figure 4. Philogenetic tree based on the partial capsid gene sequences of SaV using the Neighbor-Joining algorithm. Bootstrap values (greater than 70 %) are shown at each node as percentages of 1,000 replicates. Reference sequences are in boldface italics. Porcine enteric calicivirus (Cowden) classified as GIII was used as an outgroup. GenBank accession numbers of the reference strains used are detailed in the tree. Bar, nucleotide substitutions per site.

Figure 5. Weekly temporal distribution of the most prevalent SaV genotypes.

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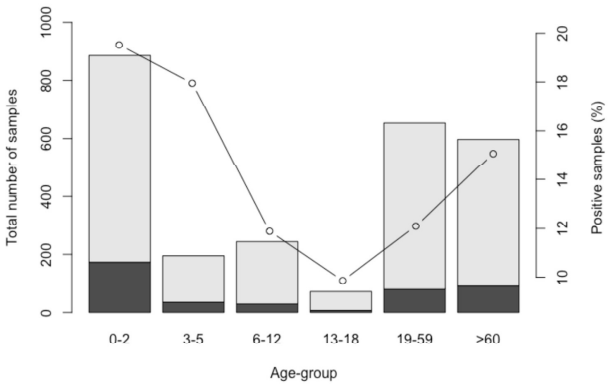

Figure 1. SaV negative (light grey) and positive samples (black), as well as human SaV prevalence within the different age-groups.

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eView

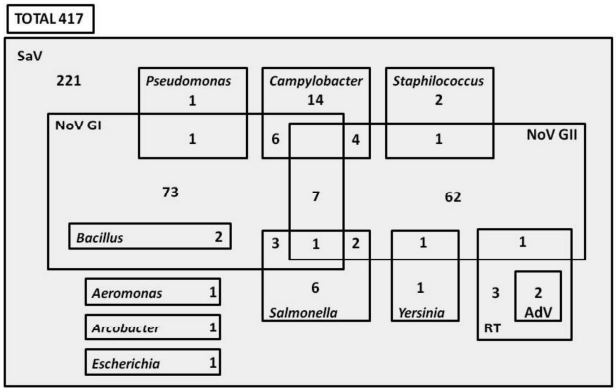

Figure 2. Diagram showing the number of SaV coinfections with other viral and bacteriological agents. Data for other pathogens obtained from previous study [10].

240x150mm (300 x 300 DPI)

For Review
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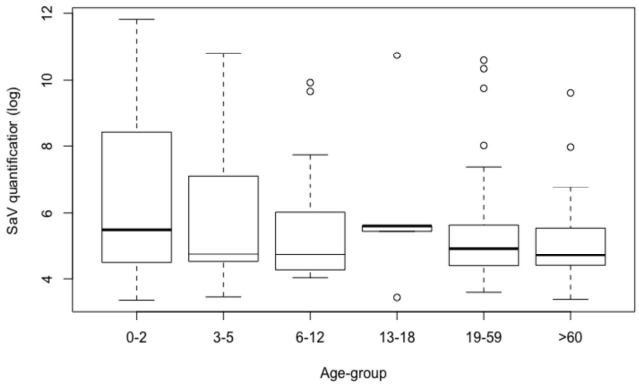

Figure 3. Boxplot showing logarithmic SaV quantifications for each age-group.

244x151mm (300 x 300 DPI)

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ReView

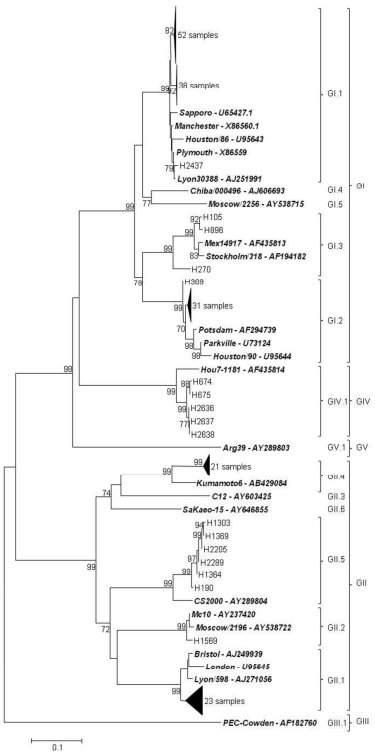

Figure 4. Philogenetic tree based on the partial capsid gene sequences of SaV using the Neighbor-Joining algorithm. Bootstrap values (greater than 70 %) are shown at each node as percentages of 1,000 replicates. Reference sequences are in boldface italics. Porcine enteric calicivirus (Cowden) classified as GIII was used as an outgroup. GenBank accession numbers of the reference strains used are detailed in the tree. Bar, nucleotide substitutions per site.

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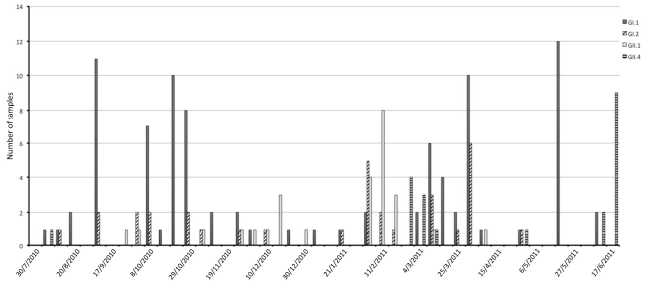

Figure 5. Weekly temporal distribution of the most prevalent SaV genotypes.

319x142mm (300 x 300 DPI)

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Human sapovirus among outpatients with acute gastroenteritis in Spain: a one year study

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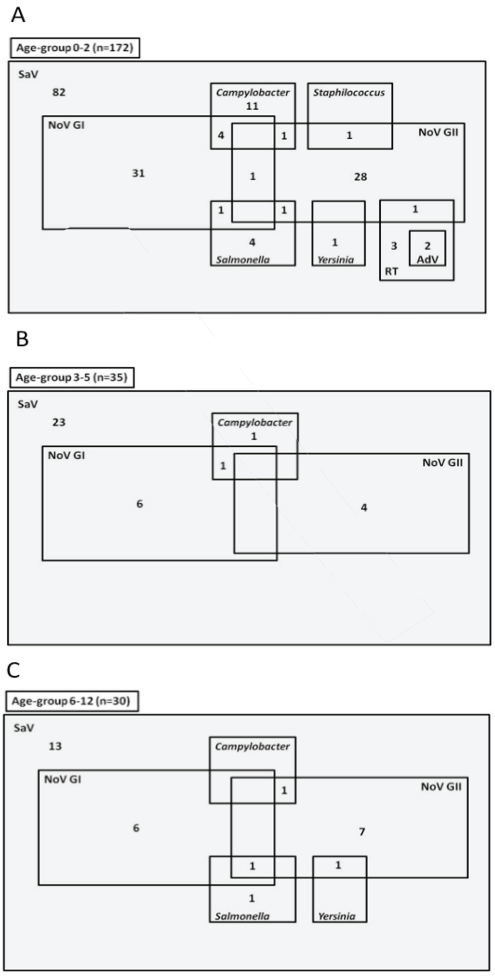
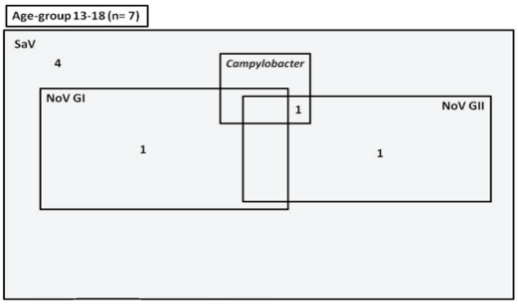
Supplementary Material

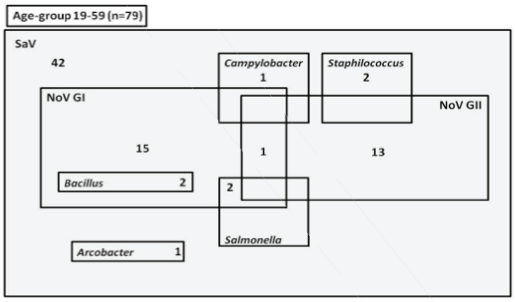
Supplementary Figure S1. Each of the 6 images (A-F) shows the number of coinfections cases of SaV with other viral and bacteriological agents for the different age-group analysed. Data for other pathogens obtained from previous study [16].



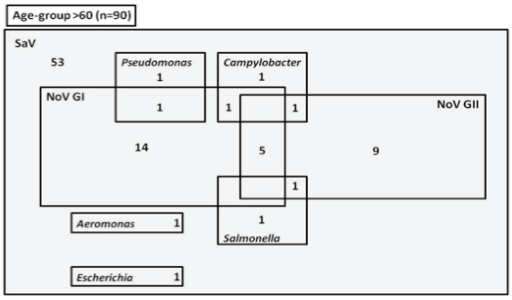
D


Age-group 13-18 (n= 7)
SaV
4
Campylobacter
NoV GI
1
NoV GII
1
1

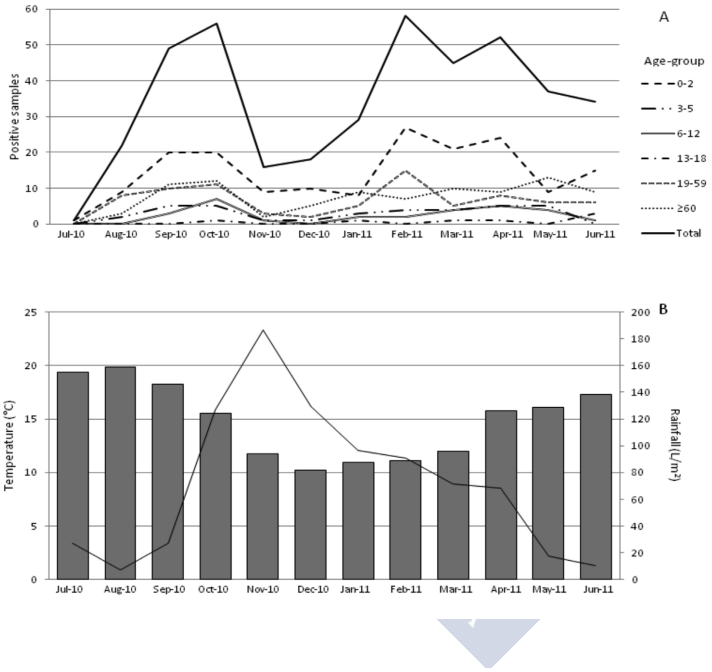
E


Age-group 19-59 (n=79)
SaV
42
Campylobacter
1
Staphilococcus
2
NoV GI
NoV GII
15
1
13
Bacillus
2
2
Salmonella
Arcobacter
1

F


Age-group >60 (n=90)
SaV
53
Pseudomonas
1
Campylobacter
1
NoV GI
1
1
1
NoV GII
14
5
9
1
Aeromonas
1
1
Salmonella
Escherichia
1

Supplementary Figure S2. (A) Positive samples in the different age-groups during the study. (B) Monthly rainfall (L/m^2) (line) and environmental temperatures (Celsium degrees) (bars) obtained from www.meteogalicia.es.



Supplementary Figure S3. Similarity matrices obtained by MegAlign of the sequence analysis for each of the genotype upturns.

GI.1 - 10/SEP/10

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	1	H334
2	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	2	H336
3	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	3	H341
4	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	4	H342
5	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	5	H344
6	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	6	H345
7	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	7	H346
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	8	H347
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	9	H348
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	10	H349
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		11	H365
	1	2	3	4	5	6	7	8	9	10	11		

GI.1 - 8/OCT/10-29/OCT/10

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		
1		100.0	100.0	100.0	100.0	100.0	100.0	99.8	99.8	99.8	99.8	99.8	99.8	100.0	99.5	99.8	100.0	99.5	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	1	H549
2	0.0		100.0	100.0	100.0	100.0	100.0	99.8	99.8	99.8	99.8	99.8	99.8	100.0	99.5	99.8	100.0	99.5	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	2	H552
3	0.0	0.0		100.0	100.0	100.0	100.0	99.8	99.8	99.8	99.8	99.8	99.8	100.0	99.5	99.8	100.0	99.5	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	3	H558
4	0.0	0.0	0.0		100.0	100.0	100.0	99.8	99.8	99.8	99.8	99.8	99.8	100.0	99.5	99.8	100.0	99.5	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	4	H545
5	0.0	0.0	0.0	0.0		100.0	100.0	99.8	99.8	99.8	99.8	99.8	99.8	100.0	99.5	99.8	100.0	99.5	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	5	H562
6	0.0	0.0	0.0	0.0	0.0		100.0	99.8	99.8	99.8	99.8	99.8	99.8	100.0	99.5	99.8	100.0	99.5	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	6	H543
7	0.0	0.0	0.0	0.0	0.0	0.0		99.8	99.8	99.8	99.8	99.8	99.8	100.0	99.5	99.8	100.0	99.5	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	7	H550
8	0.2	0.2	0.2	0.2	0.2	0.2	0.2		99.5	99.5	99.5	99.5	99.5	99.8	99.3	99.5	99.8	99.3	99.8	99.8	99.8	99.5	99.8	99.8	99.8	99.8	8	H606
9	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.5		99.5	100.0	100.0	100.0	99.8	99.8	100.0	99.8	99.8	99.8	99.8	99.8	99.5	99.8	99.8	99.8	99.8	9	H660
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2		99.5	99.5	99.5	99.8	99.3	99.5	99.8	99.3	99.8	99.8	99.8	99.5	99.8	99.8	99.8	99.8	10	H655
11	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.5	0.0	0.2		100.0	100.0	99.8	99.8	100.0	99.8	99.8	99.8	99.8	99.8	99.5	99.8	99.8	99.8	99.8	11	H662
12	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.5	0.0	0.2	0.0		100.0	99.8	99.8	100.0	99.8	99.8	99.8	99.8	99.8	99.5	99.8	99.8	99.8	99.8	12	H667
13	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.5	0.0	0.2	0.0	0.0		99.8	99.8	100.0	99.8	99.8	99.8	99.8	99.8	99.5	99.8	99.8	99.8	99.8	13	H669
14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.2	0.2	0.2		99.5	99.8	100.0	99.5	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	14	H658
15	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.7	0.2	0.5	0.2	0.2	0.2	0.5		99.8	99.5	99.5	99.5	99.5	99.5	99.3	99.5	99.5	99.5	99.5	15	H665
16	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.5	0.0	0.2	0.0	0.0	0.0	0.2	0.2		99.8	99.8	99.8	99.8	99.8	99.5	99.8	99.8	99.8	99.8	16	H661
17	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.2	0.2	0.2	0.0	0.5	0.2		99.5	100.0	100.0	100.0	99.8	100.0	100.0	100.0	100.0	17	H656
18	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.7	0.2	0.5	0.2	0.2	0.2	0.5	0.5	0.2	0.5		99.5	99.5	99.5	99.8	99.5	99.5	99.5	99.5	18	H671
19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.2	0.2	0.2	0.0	0.5	0.2	0.0	0.5		100.0	100.0	99.8	100.0	100.0	100.0	100.0	19	H714
20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.2	0.2	0.2	0.0	0.5	0.2	0.0	0.5	0.0		100.0	99.8	100.0	100.0	100.0	100.0	20	H726
21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.2	0.2	0.2	0.0	0.5	0.2	0.0	0.5	0.0	0.0		99.8	100.0	100.0	100.0	100.0	21	H713
22	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.5	0.5	0.2	0.5	0.5	0.5	0.2	0.7	0.5	0.2	0.2	0.2	0.2	0.2		99.8	99.8	99.8	99.8	22	H720
23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.2	0.2	0.2	0.0	0.5	0.2	0.0	0.5	0.0	0.0	0.0	0.2		100.0	100.0	100.0	23	H717
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.2	0.2	0.2	0.0	0.5	0.2	0.0	0.5	0.0	0.0	0.0	0.2	0.0		100.0	100.0	24	H715
25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.2	0.2	0.2	0.0	0.5	0.2	0.0	0.5	0.0	0.0	0.0	0.2	0.0	0.0		100.0	25	H718
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.0	0.2	0.2	0.2	0.0	0.5	0.2	0.0	0.5	0.0	0.0	0.0	0.2	0.0	0.0	0.0		26	H723
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		

GI.1 - 4/MAR/11-1/APR/11

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
1		100.0	100.0	100.0	99.7	99.7	99.7	100.0	100.0	99.7	100.0	100.0	100.0	100.0	97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	1	H1708
2	0.0		100.0	100.0	99.7	99.7	99.7	100.0	100.0	99.7	100.0	100.0	100.0	100.0	97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	2	H1713
3	0.0	0.0		100.0	99.7	99.7	99.7	100.0	100.0	99.7	100.0	100.0	100.0	100.0	97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	3	H1777
4	0.0	0.0	0.0		99.7	99.7	99.7	100.0	100.0	99.7	100.0	100.0	100.0	100.0	97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	4	H1778
5	0.0	0.0	0.0	0.0		99.4	99.4	99.7	99.7	99.4	99.7	99.7	99.7	99.7	97.6	97.6	97.3	96.4	97.3	99.7	99.1	99.4	99.7	99.4	5	H1782
6	0.3	0.3	0.3	0.3	0.3		99.4	99.7	99.7	99.4	99.7	99.7	99.7	99.7	97.6	97.6	97.3	96.4	97.3	99.7	99.1	99.4	99.7	99.4	6	H1783
7	0.3	0.3	0.3	0.3	0.6			99.7	99.7	99.4	99.7	99.7	99.7	99.7	97.6	97.6	97.3	96.4	97.3	99.7	99.1	99.4	99.7	99.4	7	H1784
8	0.0	0.0	0.0	0.0	0.0	0.3	0.3		100.0	99.7	100.0	100.0	100.0	100.0	97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	8	H1785
9	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0		99.7	100.0	100.0	100.0	100.0	97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	9	H1870
10	0.3	0.3	0.3	0.3	0.3	0.6	0.6	0.3	0.3		99.7	99.7	99.7	99.7	97.6	97.6	97.3	96.4	97.3	99.7	99.7	99.4	99.7	99.4	10	H1894
11	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.3		100.0	100.0	100.0	97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	11	H1935
12	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.3	0.0		100.0	100.0	97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	12	H1936
13	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.3	0.0	0.0		100.0	97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	13	H1939
14	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.3	0.0	0.0	0.0		97.9	97.9	97.6	96.7	97.6	100.0	99.4	99.7	100.0	99.7	14	H1944
15	2.1	2.1	2.1	2.1	2.1	2.4	2.4	2.1	2.1	2.4	2.1	2.1	2.1	2.1		100.0	99.7	98.8	99.7	97.9	97.9	98.2	97.9	97.6	15	H2049
16	2.1	2.1	2.1	2.1	2.1	2.4	2.4	2.1	2.1	2.4	2.1	2.1	2.1	2.1	0.0		99.7	98.8	99.7	97.9	97.9	98.2	97.9	97.6	16	H2050
17	2.4	2.4	2.4	2.4	2.4	2.7	2.7	2.4	2.4	2.7	2.4	2.4	2.4	2.4	0.3	0.3		99.1	100.0	97.6	97.6	97.9	97.6	97.3	17	H2052
18	3.3	3.3	3.3	3.3	3.3	3.6	3.6	3.3	3.3	3.6	3.3	3.3	3.3	3.3	1.2	1.2	0.9		99.1	96.7	96.7	97.0	96.7	96.4	18	H2053
19	2.4	2.4	2.4	2.4	2.4	2.7	2.7	2.4	2.4	2.7	2.4	2.4	2.4	2.4	0.3	0.3	0.0	0.9		97.6	97.6	97.9	97.6	97.3	19	H2055
20	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.3	0.0	0.0	0.0	0.0	2.1	2.1	2.4	3.3	2.4		99.4	99.7	100.0	99.7	20	H2057
21	0.6	0.6	0.6	0.6	0.6	0.9	0.9	0.6	0.6	0.3	0.6	0.6	0.6	0.6	2.1	2.1	2.4	3.3	2.4	0.6		99.1	99.4	99.1	21	H2060
22	0.3	0.3	0.3	0.3	0.3	0.6	0.6	0.3	0.3	0.6	0.3	0.3	0.3	0.3	1.8	1.8	2.1	3.0	2.1	0.3	0.9		99.7	99.4	22	H2066
23	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.3	0.0	0.0	0.0	0.0	2.1	2.1	2.4	3.3	2.4	0.0	0.6	0.3		99.7	23	H2030
24	0.3	0.3	0.3	0.3	0.3	0.6	0.6	0.3	0.3	0.6	0.3	0.3	0.3	0.3	2.4	2.4	2.7	3.6	2.7	0.3	0.9	0.6	0.3		24	H2048
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		

GII.4 - 10/JUN/11-17/JUN/11

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11		
1		99.4	99.4	99.4	98.4	99.4	99.4	99.4	99.4	99.4	99.4	1	H2634
2	0.6		100.0	100.0	99.1	100.0	100.0	100.0	100.0	100.0	100.0	2	H2640
3	0.6	0.0		100.0	99.1	100.0	100.0	100.0	100.0	100.0	100.0	3	H2642
4	0.6	0.0	0.0		99.1	100.0	100.0	100.0	100.0	100.0	100.0	4	H2648
5	1.6	0.9	0.9	0.9		99.1	99.1	99.1	99.1	99.1	99.1	5	H2660
6	0.6	0.0	0.0	0.0	0.9		100.0	100.0	100.0	100.0	100.0	6	H2679
7	0.6	0.0	0.0	0.0	0.9	0.0		100.0	100.0	100.0	100.0	7	H2680
8	0.6	0.0	0.0	0.0	0.9	0.0	0.0		100.0	100.0	100.0	8	H2681
9	0.6	0.0	0.0	0.0	0.9	0.0	0.0	0.0		100.0	100.0	9	H2682
10	0.6	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0		100.0	10	H2687
11	0.6	0.0	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0		11	H2690
	1	2	3	4	5	6	7	8	9	10	11		

3.3. New techniques on the study of sapovirus

3.3.1. Article 4. Development of a novel digital RT-PCR method for detection of human sapovirus in different matrices

https://www.sciencedirect.com/science/article/pii/S0166093417306912?via%3Dihub

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4. DISCUSSION

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4. DISCUSSION

The past decades of research have contributed to significant advances in the field of Virology. These advances involve the improvement of techniques for the detection and characterization of non-culturable waterborne and foodborne viruses, the recognition of waterborne outbreaks caused by HAV and HEV, the consideration of RV as the single most important cause of severe children gastroenteritis and NoV as the most frequent agent of foodborne diarrhea, the characterization of other important agents of non-bacterial gastroenteritis such as AsV, SaV, AdV, and the assessment of the zoonotical transmission of some of the aforementioned agents (Bosch *et al.*, 2009). It has become also clear the inefficiency of the mechanisms currently employed for microbial control to answer the vulnerabilities that threat the food and water consumption among the globe. The absence of a standardized methodology for detection and quantification of enteric viruses on foodstuff (including shellfish and drinking water) has been solved with the implementation of the ISO 15216 (ISO, 2013; 2017).

Referring to this, all assays carried out in this doctoral thesis have followed the guidelines of that cited ISO, except for minor modifications, even though it was originally published for NoV and HAV studies. These procedures include controls to assess the extraction efficiency, in order to evaluate the possible loss of viral

load during the RNA extraction; and amplification efficiency, to estimate the presence of polymerase inhibitors in the samples and avoid false negative results. All these together brings consistence in the obtained data and provides the needed reliability and reproducibility for posterior analysis and interpretation (ISO, 2013; 2017).

One of the main objectives of this doctoral thesis was to obtain a novel perspective of the presence or absence of human SaV in shellfish species from Galicia, and to study their genetic diversity. To our knowledge, the work presented was the first study of human SaV in commercial shellfish in Europe, and to date the only systematic long-term analyses reporting all together detection, quantification and genetic characterization. The most studied species was mussel (*M. galloprovincialis*) due to its importance among the aquaculture sector in this region, but some other bivalves were included for a broader view.

Results obtained clearly show that the human SaV prevalence in Galician bivalve molluscs is very significant and persistent on time. When observing together all the studies conducted in the three *rías*, SaV was tested positive in 60 out of 248 (24.2 %) of the analysed shellfish. Mussels were the most represented species, and overall rendered positive in 44 out the 185 samples (23.8 %). Both *ría* de Vigo and *ría* do Burgo are under the enormous urban influence of the two most populated cities in Galicia (Vigo and A Coruña), and

shellfish shown contamination by SaV in 17/78 (21.8 %) and 30/80 (37.5 %), respectively. Even between these two *rías*, the different SaV prevalence could be explained by the difference in size of them (as *ría* de Vigo is more than three times the size of *ría* do Burgo), and therefore by their different hydrodynamic characteristics. Contrary to this, results in *ría* de Ares-Betanzos were not as high, reaching 13/90 positives (14.4 %). This *ría* is surrounded by rural areas, with not much influence of industries and human population. According to these observations there seems to be a positive correlation between the amount of inhabitants living on the surrounds of the *ría* and the prevalence of human SaV, although broader studies and statistical analyses should be carried out to confirm this hypothesis.

To date (June 2018), only other 7 studies have analysed the presence of SaV in shellfish samples, most of them in Japan and/or sampling sporadically, not systematically. Prevalence of SaV varies a lot, especially depending on the type of study. In general, positive ratios in similar works ranged from 7.0 (Hansman *et al.*, 2007c) to 17.6 % (Fusco *et al.*, 2017), which are much lower than those observed by us. Most of those other studies used conventional PCR protocols, and in the ones using quantification methods the viral loads were calculated between 10^2 and 10^4 GC/g of digestive tissue (Benabbes *et al.*, 2013; Tryland *et al.*, 2014; Fusco *et al.*, 2017). In our study, the viral quantification ranged between 10^3 and 10^5 GC/g of digestive tissue, which is a slightly higher quantification titer.

Genetic analyses conducted on the positive samples in our study shown a broad diversity of human SaV genotypes circulating on the Galician seawater environment. Of the 60 positive samples, genetic characterization was possible in 30 (50.0 %), which is considered a good genotyping ratio for environmental samples (Kitajima *et al.*, 2010). Genogroup I was, as expected, the most common with 25 strains, divided into GI.1 (11), GI.2 (10) and GI.3 (4); but also genogroups II (GII.4), GIV and GV were detected. Not many studies have genotyped SaV strain obtained from shellfish samples, but the diversity observed in Galician bivalve molluscs is in accordance with a study by Iizuka *et al.* (2013), who even detected different genotypes present in the same individual clam. A different genetic characterization approach than Sanger sequencing (e.g., NGS techniques or cloning) should have been employed in our studies in order to detect genotypes present in the same sample.

A study conducted in Norway showed that SaV contamination in sentinel mussels grown in urban areas occurred only after heavy rain episodes in the spring/summer season (Tryland *et al.*, 2014). But, as SaV shellfish studies are scarce, it is yet not clear the real correlation between climate conditions and their presence in shellfish samples. Attending to the seasonality observed for other environmental samples and for other enteric viruses, such as NoV, the cold seasons normally present the highest positive rate, which is

in accordance with our results (Hansman *et al.*, 2007c; Kitajima *et al.*, 2011).

The other main objective of this thesis was the study of SaV among the Galician population during a period of one year, for which their prevalence was analysed in patients suffering of acute gastroenteritis and the most common circulating strains were characterized. The work presented here brings new data on the etiology of SaV being, to our knowledge, the first long-term study of human SaV in stool samples in Spain, and one of the only ones in Europe. The lack of available epidemiological data difficult the possibility of associate these results with any outbreaks of the disease.

Results obtained shown a higher prevalence (15.6 %) than other clinical studies (Oka *et al.*, 2015), but agreed with the established notion of higher positive rate of this pathogen especially in infants and children, but also in elder patients (Wang *et al.*, 2014; Thongprachum *et al.*, 2015). Shedding levels ranging up to 10^{11} GC/g of stool (mean value of 10^{5} GC/g of stool) are also normal when quantifying these enteric viruses, as previously reported (Iwakiri *et al.*, 2009). Positive samples are partially in accordance with the seasonality described for this pathogen, indicating a high incidence during cold months (Pang *et al.*, 2000), but prevalence was even higher during autumn and spring months, and the viruses were detected in patients during the entire period of study.

The studies discussed so far can be used to compare the genotyping data obtained among them. As mentioned above, the most common genotypes obtained in Galician shellfish were GI.1 and GI.2, which were also the dominant among the acute gastroenteritis patients infected by SaV (in 91 and 32 cases, respectively). In shellfish, these genotypes represented the 83.3 % of all the genotyped strains, and in humans the 66.1 % of the 186 samples that could be genetically characterized. These two strains are the most common ones detected worldwide (Gallimore *et al.*, 2006; Hansman *et al.*, 2007d; Harada *et al.*, 2009; Svraka *et al.*, 2010; Kumthip *et al.*, 2017), but apart from them, both in the shellfish and clinical studies, genotypes GI.3, GII.4 and GIV.1 were detected, although in lower numbers. All genotypes detected in shellfish samples were also detected in infected human, except GV.1 (Figure 9). These facts points towards a clear circulated transmission route: the excretion on the environment of certain human SaV viral strains by infected people, the bioaccumulation by shellfish growing in those areas, and the human consumption of those contaminated shellfish, becoming again infected by the pathogen.

Limitations of the PCR methodologies do not make possible to establish a clear relation with the infective viruses implicated, as this technique only takes into account the amplification of nucleic acids and SaV is not culturable for infectivity experiments. Therefore, the real risk of infection for the shellfish consumer remains partially unknown.

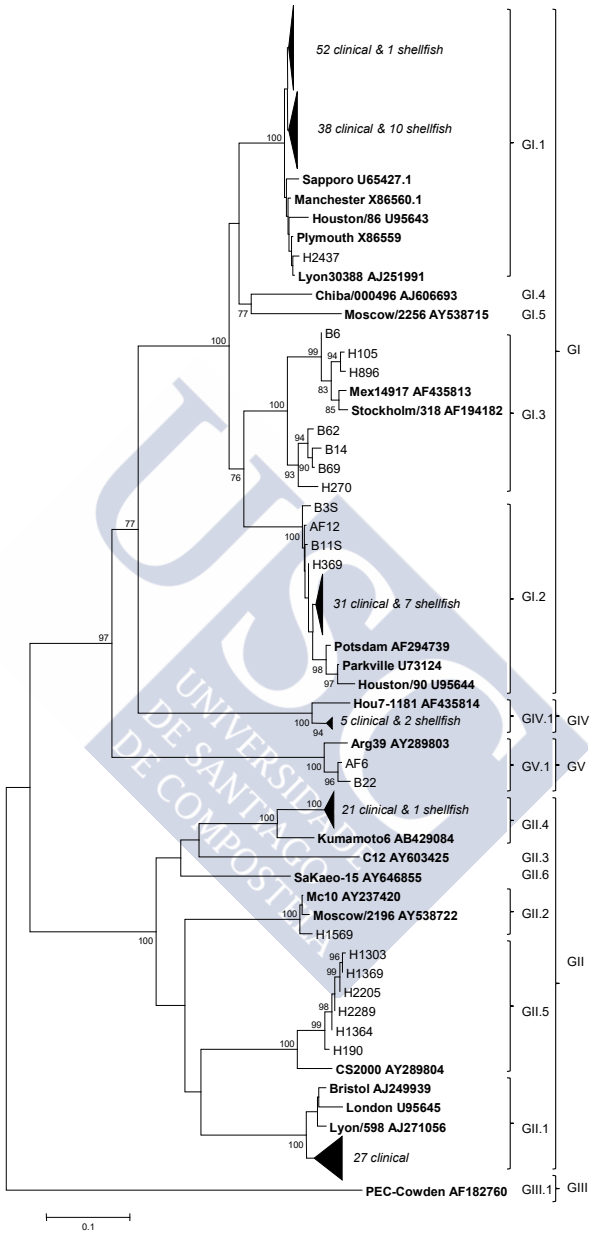

Figure 9. Phylogenetic tree constructed using the partial VP1 nucleotide sequences of all clinical and shellfish SaV strains obtained in this thesis. Representative strains of each genotype are in boldface with accession numbers, and genotypes indicated. The scale represents nucleotide substitutions per site. Clinical samples named with *H*, others are shellfish.

One of those PCR methodologies (i.e., RT-qPCR) is the most employed worldwide in enteric virus studies, in the absence of a robust cell culture system, and was the technique used for all our experimental work. The last objective on this doctoral thesis was to develop a protocol to overcome some of those limitations with the purpose of achieving more accurate results when studying SaV. A sensitive detection of enteric viruses in clinical and environmental samples is extremely relevant, especially due to the low concentrations in which they are found in food and water. A microfluidic digital PCR technique provides quantifications without the need for standardized materials, sometimes hard to obtain, and a standard curve, resulting in absolute quantification instead of relative. Inhibition on qPCR reduces the efficiency of amplification, which conducts to an underestimation or even a non detection. A higher tolerance against PCR inhibitors has been reported in digital PCR as the sample mix in partitioned in thousands of independent and parallel nano-reactions (Huggett *et al.*, 2013), and, as it is an endpoint reaction, a decreased in the amplification efficiency can still be detected providing a positive result. Indirect quantification may lead to errors with a possible overestimation of the standards (Sanders *et al.*, 2013), as qPCR can yield an estimated increase or decrease of 50 % in the target copy numbers (Sanders *et al.*, 2011). Nevertheless, the digital platform produces more accurate measurements not only for standards but also for experimental materials in different matrices, as resulted in our study.

Further and broader investigations are needed on the presence and distribution of human SaV in Galicia among shellfish and other foodstuff, as well as in wastewater and coastal sediments. Epidemiological researches could also become more complete if the proper epidemiology information available. All these together, with a more complete genetic analysis of the detected strains, could help to clarify the importance of SaV as an emerging enteric pathogen in Spain. Nevertheless, information obtained in this work reveals, once more, the importance for the consumer of settling classifications, controls and limits on the growing waters and the harvested shellfish attending to viral pathogens, and not only bacterial indicators. As said at the beginning of this thesis, *"safe food saves lives"*, and we hope that the discoveries achieved in this work could become helpful filling a certain gap of knowledge in this field of study.

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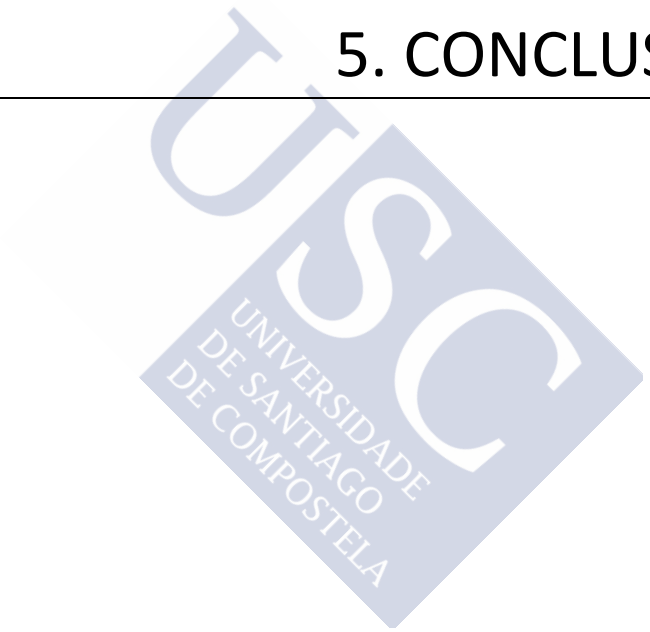
5. CONCLUSIONS



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5. CONCLUSIONS

From the results obtained along this work it can be concluded that:

1. The presence of human sapovirus was, for the first time, confirmed in Galicia (Spain), both in shellfish and in patients suffering from acute gastroenteritis.

2. The detection rate and the quantification levels observed for sapovirus in shellfish from different *rías* support the lack of reliance between the current classification criteria for shellfish harvesting waters (based only on bacterial indicators) and the presence of human enteric viruses.

3. The incidence of human sapovirus among gastroenteritis patients in the A Coruña metropolitan area revealed this pathogen as an important etiological agent in the region for all age-groups, and especially for infants and children.

4. Phylogenetic analysis conducted on shellfish and clinical samples showed a broad SaV genetic diversity circulating in Galicia with genotypes GI.1 and GI.2 as the most detected; and pointed towards what could be a transmission route.

5. The development of a new digital PCR protocol for clinical, shellfish and environmental water samples rendered great effectiveness and repetitiveness, as well as higher sensitivity than real time PCR assays, constituting a promising new tool for the study of human sapovirus.

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